

**PATHOPHYSIOLOGY OF POST-OPERATIVE  
PERITONEAL ADHESIONS**

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Doctor of Medicine**

**HASSAN SULAIMAN  
MB, BCH, MSc, FRCS, FWACS**

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**Departments of Surgery and Medicine  
Royal Free and University College London Medical School**

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## **ABSTRACT**

Post-operative peritoneal adhesions are fibrous bands of tissue joining together organs occurring in the majority of patients following laparotomy, leading to complications such as abdominal pain, infertility in women and intestinal obstruction. However the structure and pathogenesis of adhesions is still not clear. It is proposed that persistence of fibrin between damaged tissues due to impaired plasminogen activator activity induces adhesion formation. This thesis aims to: (a) assess the structure of peritoneal adhesions in humans and in an experimental murine model; (b) characterise and assess the growth of nerve fibres in adhesions; and (c) to elucidate the role of fibrinolysis in adhesion formation in animals.

Human peritoneal adhesions were collected from patients undergoing laparotomy, whereas murine adhesions were generated in a standardised murine model. Adhesion samples were processed and nerve fibres were characterized histologically, immunohistochemically, and ultrastructurally. In mice two-weeks post-operatively adhesions contained nerve fibres which were synaptophysin, calcitonin-gene-related-peptide, and substance-P-immunoreactive. At 4-weeks, nerve fibres originated from both the caecum and abdominal wall and by acetylcholinesterase-histochemistry were found traversing the entire adhesion. Nerve fibres were similarly present in all human adhesion specimens assessed. Ultrastructural analysis showed both myelinated and non-myelinated nerve fibres were present in human and mouse adhesions.

To elucidate the role of fibrinolysis a novel approach was taken using mice deficient in tissue plasminogen activator (t-PA) or urokinase plasminogen activators (u-PA) and inducing adhesion formation either by surgery or following an inflammatory episode. Mice deficient in t-PA were significantly more susceptible to adhesion formation following chronic inflammatory episode compared with u-PA deficient and wild-type mice.

This thesis provides the first direct evidence that sensory nerve fibres grow into peritoneal adhesions suggesting that they may be capable of conducting pain sensation. Furthermore the persistence of fibrin due to decreased t-PA activity plays a major role in peritoneal adhesion formation.



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## **LIST OF ABBREVIATIONS**

AW	Abdominal wall
Adh	Adhesion
b-FGF	Basic- fibroblast growth
BNF	British National Formulary
C	Caecum
CAPD	Continuous ambulatory peritoneal dialysis
CGRP	Calcitonin gene related peptide
CGRP-IR	Calcitonin gene related peptide immunoreactive
C. parvum	Corynebactrium Parvum
CT	Computerised tomography
dd	Double distilled water
ECM	Extracellular matrix
FB	Fat body
FDP	Fibrin degradation products

GAG	Glucosaminoglycan
GIT	Gastrointestinal tract
H&E	Haematoxylin and Eosin
HA	Hyaluronic acid
HA-CMC	Hyaluronic acid-Carboxymethylcellulose (Septrafilm)
HA-PBS	Hyaluronic acid-phosphate-buffered saline solution
IL	Interleukin
I.P.	Intra-peritoneal
L	Liver
LTD4	Leukotriene D4
L2	Lumbar nerve 2
MQ	Macrophages
MSC	Mesenchymal stem cell
NGF	Nerve growth factor
ORC	Oxidized regenerated cellulose (Interceed)
PAI	Plasminogen activator inhibitor
PBS	Phosphate-buffered saline
PDGF	Platelet derived growth factor
PMNL	Polymorphnuclear leucocyte
PTFE	Polytetrafluoroethylene (Gore-Tex)
RBC	Red blood cell
S	Schwann's cell
S B	Small bowel
scu-PA	Single chain urokinase plasminogen activator
sc-t PA	Single chain tissue plasminogen activator



<b>SEM</b>	<b>Scanning electron microscopy</b>
<b>S-P</b>	<b>Substance P</b>
<b>S P-IR</b>	<b>Substance P-immunoreactive</b>
<b>SYN</b>	<b>Synaptophysin</b>
<b>SYN-IR</b>	<b>Synaptophysin-immunoreactive</b>
<b>T5</b>	<b>Thoracic nerve 5</b>
<b>tc- tPA</b>	<b>Two chain tissue plasminogen activator</b>
<b>TEM</b>	<b>Transmission electron microscopy</b>
<b>T-H</b>	<b>Tyrosine hydroxylase</b>
<b>T H-IR</b>	<b>Tyrosine hydroxylase-immunoreactive</b>
<b>TGF-<math>\alpha</math></b>	<b>Transforming growth factor-alpha</b>
<b>TGF-<math>\beta</math>1</b>	<b>Transforming growth factor-beta 1</b>
<b>TNF- <math>\alpha</math></b>	<b>Tumour necrosis factor-alpha</b>
<b>t PA</b>	<b>Tissue plasminogen activator</b>
<b>VEGF</b>	<b>Vascular endothelial growth factor</b>
<b>V I P</b>	<b>Vasointestinal peptide</b>
<b>V I P-IR</b>	<b>Vasointestinal peptide-immunoreactive</b>
<b>VPF</b>	<b>Vascular permeability factor</b>
<b>uPA</b>	<b>Urokinase plasminogen activator</b>
<b>u-PAR</b>	<b>Urokinase plasminogen activator receptor</b>

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## **CHAPTER ONE**

### **INTRODUCTION**

## **1.1 BACKGROUND**

Peritoneal adhesion formation involves the abnormal attachment of normally separated abdominal/pelvic organs or serosal surfaces by bands of fibrous tissue. Adhesions are the result of damage to serosal surface mainly due to surgical intervention, however, infection within the peritoneal cavity, dryness of serosal surfaces, presence of foreign bodies and radiation can also cause adhesions. Subsequent complications of adhesions include chronic abdominal pain, infertility in women and the more serious, intestinal obstruction with its fatal consequences. Adhesions may result from events occurring during fetal development (congenital adhesions) in 3-11% of humans (Perry, 1955). Adhesions also occur at other sites such as the pleural and pericardial cavities, tendon sheaths and conjunctiva. Post-operative peritoneal adhesions and their complications have a great impact on patients' welfare and health service resources. However, the mechanisms of adhesion formation are not yet fully understood and have not been investigated at the cellular and molecular level. Many preventive or therapeutic measures have been devised but are far from fulfilling their objective.

In the last century, knowledge has been accumulated regarding the structure and function of the serosal membranes. A publication by Bichat in 1827 was the first to record that serous cavities are covered by a layer of flattened cells. The term "mesothelium" was initially used by Minot in 1890 while performing embryological studies he noted that "the epithelial lining of the so called mesodermal cavity was a flattened monolayer" and linking its morphological similarity to epithelium and its origin from the mesoderm he proposed the term "mesothelium". In 1872, Bryant described a case of fatal intestinal obstruction resulting from adhesions to a ligature after an ovarian cystectomy and Battle in 1883 reported intestinal obstruction due to formation of dense adhesions following removal of bilateral ovarian cysts. In his book on the peritoneum published in 1919 Hertzler summarised much of the current knowledge regarding serous

membrane structure and function. This included histological studies of mesothelial regeneration and the first observation of spindle-shaped cells at the site of injury leading to the assumption that the new surface mesothelium might be derived from “submesothelial fibroblasts”. With a increase in surgical intervention the impact of peritoneal adhesions has come well recognised.

## **1.2 CLINICAL IMPACT OF PERITONEAL ADHESIONS**

Post-operative peritoneal adhesions occur in 67-100% of patients undergoing laparotomy (Luijendijk et al., 1996). In two large surveys it was found that there was no difference in incidence between male and female patients after excluding previous gynaecological procedures and adhesion formation did not appear to be age dependent (Perry et al., 1955; Weibel and Majno, 1973). Indeed neonates are not immune from this problem (Wilkins and Spitz, 1986). Peritoneal adhesion occurs between any two sites, the commonest being omentum and incision scar followed by site of surgical procedure and incision scar, omentum and site of surgery; small bowel and incision scar, small bowel and site of surgery, small bowel and small bowel (Menzies and Ellis, 1990).

Most adhesions are usually symptom-less, however they can lead to major complications such as infertility in women, intestinal obstruction and recurrent abdominal pain as detailed in the following section (Gomel, 1983).

The role of peritoneal adhesions in the aetiology of infertility has been well documented and infertility in approximately 30% of women is caused by adhesions (Drake, 1980; DeCherney and Merzer, 1984). Adhesions are responsible for failure of reconstructive tubal surgery demanding second look laparoscopy and adhesiolysis. Newly formed adhesions are mechanically lysed at an early stage when they are still gelatinous and easily separable. A conception rate of between 39% and 63% has been reported following lysis of pelvic (periadnexal) adhesions (Diamond, 1987).



Adhesions are the commonest cause of intestinal obstruction with some particularly following appendicectomy and large bowel surgery. Stewart et al. (1987) found that postoperative obstruction occurred within 4 weeks in 0.69% of patients who had undergone laparotomy, with over 90% of cases directly due to adhesions. In a prospective 10 years follow up study (SCAR study) in Scotland, of approximately 30,000 patients who underwent abdominal or pelvic procedures, 34.7% required one or more readmissions for surgical or medical treatment for conditions either related to adhesions (5.5% directly related) or involving a re-operation that could be complicated by adhesions (Ellis et al., 1999). Raf et al. (1969) in Stockholm analysed 1477 cases of intestinal obstruction due to adhesions and found that 86% of these followed abdominal surgery, appendicectomy in 38% and gynaecological operations in 28% of cases. Other studies have shown that the incidence of intestinal obstruction due to adhesions may be as high as 65-75% (Ellis, 1998; Thompson and Whawell, 1995) with a considerable risk up to 20 years after an episode of adhesive small bowel obstruction (Bjorg-Tilde et al., 2004). Unfortunately, in a substantial number of patients adhesions rapidly reform following adhesiolysis. For example, one Australian study showed a 45% - 80% recurrence rate and an intestinal obstruction rate possibly as high as 32% (Jansen, 1988). Of interest, in communities that lack the medical facilities for interventional abdominal surgery, strangulated hernia rather than adhesions, accounts for the majority of cases of intestinal obstruction (McAdam, 1961)

Chronic abdominal/pelvic pain, of more than six months duration is a common postoperative complaint (Kresch et al., 1984; Howard, 1994; Keltz et al., 1995; Popora and Gomel, 1997), believed to be caused by adhesions and adhesiolysis is the only effective therapeutic approach (Goldstein et al., 1980; Steege and Stout, 1991; Nezhat et al., 1990), although this is controversial. In a study by Sutton and MacDonald (1990) 65 patients with chronic pelvic pain underwent laser laparoscopic adhesiolysis with

symptomatic relief in 84% of patients with a follow up of 1-5 years. Several studies reported similar results (Daniel, 1989; Steege and Stout, 1991; Fayez and Clarke, 1994), although others have disputed an association between abdominal/pelvic pain and adhesions (Rapkins, 1986; Alexander-Williams, 1987).

### **1.3 ADHESIONS AND THE COST TO HEALTHCARE**

Post-operative abdominal adhesions present a problem for the health services and a burden on its limited financial resources. A study of 28,000 adult surgical admissions at Westminster Hospital, London, over a 24 year period (1964-1988) found that adhesive intestinal obstruction accounted for 0.9% of all admissions, 3.3% of the 4,502 major laparotomies, and 28.8% of the 514 admissions for large or small bowel obstruction (Menzies and Ellis, 1990). In general surgery alone adhesions may create or complicate up to 18,000 re-admission cases per year in the UK. Furthermore the mean duration of hospitalization for British patients who have undergone an operation for adhesive obstruction exceeds 15 days (Mc Entee et al., 1987). A similar study suggested that the different modalities of treatment for adhesive obstruction whether conservative management or interventional approach do not affect the frequency of re-admissions for the same problem with an increasing cost (Wilson et al., 1998). In the USA Ray et al. (1994) described the number of hospitalizations and days of care attributable to adhesiolysis. There were 303,836 hospitalizations, and 19% of these were for primary adhesiolysis, while the remaining 81% included secondary adhesiolysis. The overall cost for hospitalization and surgeons' fees was US \$1.3 billion. Holmdahl and Risberg (1997), estimated that adhesions resulted in 4,500 laparotomies in Sweden and SEK 130 million (US \$ 20 million dollars) in public health expenditures.

### **1.4 DIAGNOSIS OF PERITONEAL ADHESIONS**

Peritoneal adhesions can cause a variety of abdominal symptoms that can not be definitively diagnosed by non-invasive methods. In general diagnosis can be made

either under conscious sedation via small diameter laparoscopy or under general anaesthesia (laparoscopy/laparotomy). Ultrasound scanning has been used to demonstrate adherence of bowel loops to the abdominal wall and can be used to assess the movement of the viscera against the parietal peritoneum (*visceral slide test*) in defining the presence of adhesions to the anterior abdominal wall (Sigel et al., 1991). With longitudinal ultrasound scanning normal spontaneous visceral slide (produced by respiratory movement) ranges from 2 to 5 cm or occasionally more in distance. During either longitudinal or transverse scanning visceral slide produced by manual compression (manual graded compression) usually exceed 1 cm. Restricted visceral slide occurs when there is an ultrasonically detected reduction in visceral slide excursion of less than 1 cm for both spontaneous and induced visceral slide (Kodama et al., 1992). In recent years attention has focused on the potential of computed tomography (CT) for diagnosis of gastro-intestinal disease and management of adhesive small bowel obstruction (Donckier et al., 1998). The technique is non-invasive and provides a complete image of the abdomen (Balthazar et al., 1992). New methods such as CT-angiography and virtual endoscopy have developed and as cross-sectional methods provide optimal tissue contrast resolution and have the advantage of visualization without superposition they are likely to play a major role in future imaging of peritoneal adhesions. Magnetic resonance imaging (MRI) of the gastro-intestinal tract has been mainly used for the diagnosis of inflammatory bowel disease, neoplasms and the detection of areas of ischaemia. However its value for peritoneal adhesions has not been explored.

It is evident that adhesions and their complications impose a massive strain on health service resources, and most efforts, techniques and funds are directed toward treatment of symptomatic adhesions rather than their prevention. To further understand

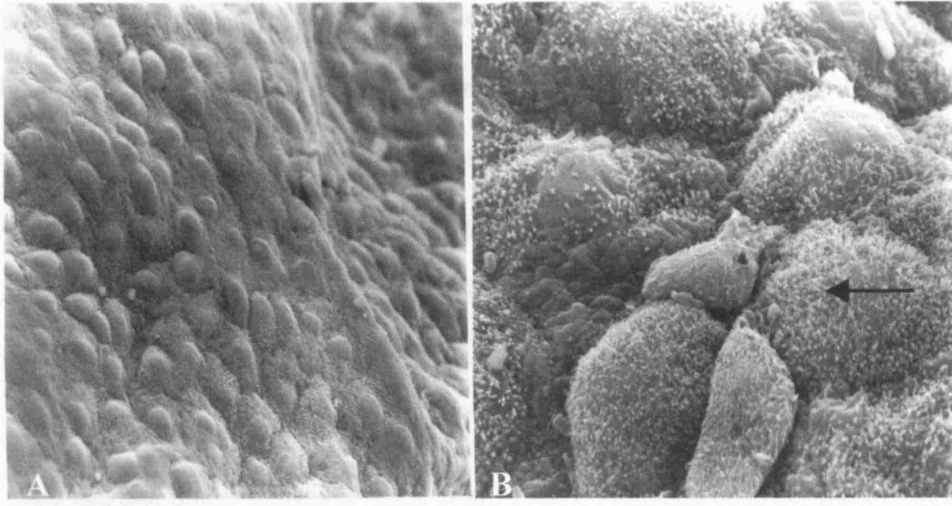
the mechanisms leading to adhesion formation, our current knowledge of the peritoneum and visceral organs is presented in the following section.

## **1.5 THE PERITONEUM**

### **1.5.1 Anatomy of the peritoneum**

The peritoneum is composed of two layers: (a) the parietal peritoneum that lines the anterior and posterior abdominal body wall and (b) the visceral peritoneum that is reflected over the internal organs and is continuous with their fibrous tissue stroma. The peritoneum is the largest and most complex serosal membrane in the body. With a surface area of approximately 10,000 cm<sup>2</sup> in adults (Esperanca and Collins, 1966), almost equal to that of the skin, this membrane may be the largest organ in the human body and it is one of the most richly vascularized organs. It forms a closed sac in the male and an open sac in the female, as the ends of the fallopian tubes and ovaries are not sealed by peritoneum (Di Zerega, 2000). The surface lining of the peritoneum consists of a layer of highly differentiated mesothelial cells (Figure 1.1). Other sites where such cells occur are the pleura, pericardium and the tunica vaginalis of the testis. The mesothelium extends as a monolayer of elongated flattened squamous cells approximately 25µm in diameter and 3 µm in thickness over the entire peritoneal surface. These cells contain a well-developed cytoskeleton of intermediate filaments, an abundant endoplasmic reticulum and Golgi apparatus as well as numerous smooth and coated vesicles indicative of active transmembrane transport (Slater, 1989).

Numerous microvilli up to 3 µm in length (Figure 1.2), varying in shape and density project from the apical surface of the mesothelial cells to minimise the shear between contiguous surfaces (Natakani, 1996). They are more numerous on the visceral than on the parietal surface reflecting a functional adaptation.



**Figure 1.1** (A) Scanning electron micrograph of murine mesothelial cells on the surface of the parietal peritoneum (x500); (B): Higher magnification showing microvilli on the cell surface. (arrow) (x800).



**Figure 1.2** Transmission electron micrograph of a mesothelial cell with microvilli on the cell surface (arrow) (x37000).

There is an association between microvillar formation and surface charge, in protecting the healing mesothelium by enhancing entrapment of serosal fluid and its contents (Mutsaers et al., 1996). Over the surface mesothelial lining is a negatively charged glycocalyx film 5 $\mu$ m thick that contains significant amounts of

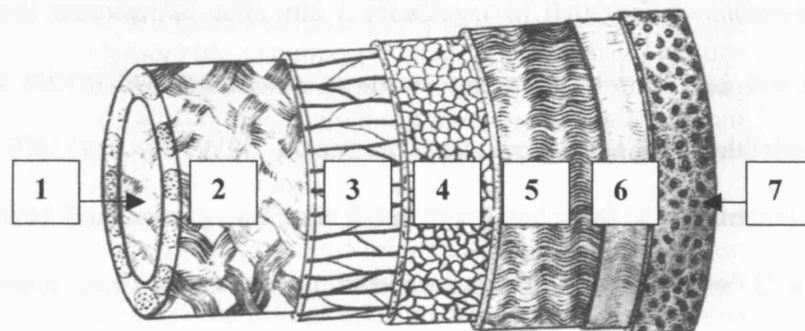
glucosaminoglycans and phospholipids in the form of phosphatidylcholine, phosphatidylethanolamine and sphingomyelin (De Paolo et al., 1986). The glycocalyx trapped between the microvilli together with the amorphous material in the intercellular spaces and the dense basement membrane act as a filter (Slater, 1989). Adjacent mesothelial cells are attached together by tight junctions close to their luminal surface, desmosomes, cadherins and intercellular canaliculi (Takashani, 1991).

The diaphragmatic mesothelium contains two morphologically distinct cell types, flattened and dome-shaped (French et al., 1960). Flattened cells are tightly juxtaposed by occludin junctions, while dome-shaped cells often surround stomata or mesothelial pores that open into lymphatic vessels (Leak, 1978) (Figure 1.3). Stomata are present at sites where the margins of several lymphatic endothelial cells span the submesothelial connective tissue. This arrangement creates a passageway between peritoneal cavity and lymphatic lumen to allow influx and efflux of cells and fluids.



**Figure 1.3:** Scanning electron micrograph showing stomata within the diaphragmatic peritoneum. Note the orifice of a lymphatic vessel (arrow) (x1000).

Mesothelial cells rest on a basement membrane that consists of a 40-70 nm layer that is absent at multiple sites such as the omentum, mesentery and diaphragm to allow for the passage of particulate matter (Mutsaers, 2002). The mesothelium covers a submesothelial layer consisting of a delicate fibrillar plexus of reticular connective tissue arranged into bundles in a plane parallel to the serosal surface. Submesothelial tissue consists of glycosylated proteins including glycosaminoglycans, collagen and elastin (Di Zerega, 2000). The peritoneal elastic lamina, like the elastic tissue in other serosal cavities, is a well-defined network of elastic fibres lying in the submesothelial layer (Figure 1.4) that gives the peritoneum mobility. The submesothelial layer contains cellular components such as fibroblasts, macrophages, lymphocytes, plasma cells, eosinophils, mast cells and adipocytes.



**Figure 1.4:** Diagrammatic presentation of visceral peritoneum and underlying layers by a transverse section of the bowel. 1: mucosal lining; 2: collagen; 3: blood vessels; 4: GAGs/mucopolysaccharide layer; 5: elastin; 6: connective tissue matrix; 7: mesothelial cells. (Modified from Baron, 1941).

Interspersed among the connective tissue matrix are extremely poorly differentiated epitheloid-like cells known as the undifferentiated mesenchymal cells (Ellis et al., 1965). These cells can undergo a variety of morphological changes into mesothelial cells or a number other cellular components including macrophages and fibroblasts after exposure to injury or other types of stimuli (Raftery, 1973).

### **1.5.2 Development of the peritoneum**

During foetal development in humans, the peritoneum develops from the lateral plate mesoderm that divides into somatic and splanchnic mesodermal layers. By the end of the third week of gestation, the parietal peritoneum develops from the somatic mesoderm while the visceral peritoneum develops from the splanchnic mesoderm. These are the borders of the intra-embryonic coelom (the future body wall). The parietal mesoderm and the overlying ectoderm form the ventral and lateral body wall by week four, while the visceral mesoderm covers the visceral organs. The mesoblast cells adjacent to the coelom are transformed into mesothelium (Moore and Dalley, 1999). Ultrastructural studies of mesothelial cell differentiation in several species have traced the gradual transformation of these undifferentiated, isolated, and spindle-shaped mesodermal cells into a monolayer of flattened epithelium-like cells with surface microvilli resting on a basement membrane and forming cell junctions (Tiedman, 1976; Ivanova, 1975). Mesothelial cells are distinct from epithelial cells as they are derived from mesodermal cells rather than ectodermal or endodermal elements and they retain some characteristic mesodermally derived features. Cytoskeleton intermediate filaments are mesenchymal type (vimentin) rather than keratin filaments as found in epithelia proper.

### **1.5.3 Blood Supply**

Due to different developmental pathways the parietal and visceral layers of the peritoneum are supplied by different circulatory networks. The parietal peritoneum is supplied by somatic blood vessels of the abdominal and pelvic wall, such as the superior and inferior epigastric arteries as well as the collateral branches of intercostal arteries. The visceral peritoneum as an integral part of the viscera derives its blood supply from the vessels supplying the visceral organs. Venous drainage is through the venous channels accompanying the arteries.



#### **1.5.4 Lymphatic Drainage**

The lymphatic drainage is mainly through the diaphragm via intrinsic lymphatics to the collecting lymphatics beneath the diaphragmatic pleura. The mesothelial cells lining the muscular portion of the diaphragm are interrupted by a large number of intermesothelial gaps (stomata). Absence of the basement membrane allows the stomata to communicate directly with the underlying lymphatic lacunae (Nakatani et al., 1996). Lymph flows from the stomata into networks under the diaphragmatic pleura and into the main lymphatic ducts via the substernal lymph nodes (Abu-Hijleh et al., 1995; Li and Jiang, 1993). Under normal circumstances about one third of the fluid draining from the peritoneal cavity passes through diaphragmatic lymphatics while the remainder exits through pores/stomata (Flessner, 1983). The mesentery has been described as having a simple type of stomata with patent portals on either side of the mesentery connecting the peritoneal cavity with underlying lymphatics (Ettar and Carr, 1996).

#### **1.5.5 Nerve Supply**

The peritoneum has a dual nerve supply, somatic for the parietal peritoneum and autonomic or visceral afferent for the visceral peritoneum. Branches of the spinal somatic nerves (T5-L2) that supply the abdominal wall innervate the parietal peritoneum and are rich in pain receptors. The visceral peritoneum receives its nerve supply from the underlying organs and these are derived from the autonomic nervous system and the enteric nervous system (Germann and Stanfield, 2005).

The gastrointestinal tract has its own intrinsic nerves “the enteric nervous system”, which lies entirely in the wall of the gut and is linked to, but separate from extrinsic autonomic nerves. It is composed mainly of two ganglionated plexuses: (1) an outer plexus lying between the longitudinal and circular muscle layers called myenteric or Auerbach’s plexus and (2) an inner plexus that lies in the submucosa called

submucosal or Meissner's plexus. This intrinsic nervous system of the gut is connected with extrinsic autonomic nerves but can also function independently (Gabella, 1991).

Differences in the sensitivity of the two layers correlate with the type and amount of innervation. Whereas pain is elicited by mechanical, thermal or chemical stimulation of the parietal peritoneum, the visceral peritoneum and viscera are not responsive to such stimuli. For example, the liver, stomach or intestine can be injured without evoking pain sensation, insensibility extends from the mid-oesophagus to the junction of endoderm and ectoderm in the anal canal. However tension does evoke pain by over-distension, traction or stretching various neural elements in the visceral wall or mesentery due to the abundance of stretch receptors. The parietal peritoneum lining the diaphragm is supplied with afferent fibres by the phrenic nerves centrally and peripherally by the lower six intercostal and subcostal nerves. Peripheral irritation results in pain, tenderness and muscular rigidity in the distribution of the lower thoracic spinal nerves, while central irritation results in pain sensation in the subcutaneous distribution of the third to fifth cervical spinal nerves or the shoulder region (Germann and Stanfield, 2005).

#### **1.5.6 Functions of the peritoneum**

The peritoneum has many different functions, the most important being to provide a protective barrier to the viscera and a frictionless interface for the free movement of apposing visceral tissues. The presence of peritoneal fluid and surfactant coating mesothelial cells aids this process. Free movement is required for normal function of the gastro-intestinal tract such as peristalsis and defaecation, bladder voiding and in the female genital tract, tubal motility and oocyte pickup (Haney, 2000).

A role for mesothelial microvilli in transport function and movement of fluids and particulate matter across the serosal cavities has been suggested due to its absorptive and exudative ability (Andrew and Porter, 1973). This property has been

applied in continuous ambulatory peritoneal dialysis (CAPD) for patients with chronic renal failure. The peritoneum plays a major role in the resistance and localization of infection within the peritoneal cavity (Hall et al., 1998). Mesothelial cells play a role in the inflammatory process by acting as antigen presenting cells and in the production of growth factors, cytokines, and protease inhibitors (Mutsaers et al., 1997).

### **1.5.7 Peritoneal Fluid**

Peritoneal fluid primarily represents a transudate across the peritoneal membrane (Koninckx et al., 1980; Maathius et al., 1978). Biologically active molecules can enter the peritoneal cavity via transudation from blood vessels, exudation, facilitated transport, by the lymphatics or can be secreted by leucocytes and mesothelial cells in the peritoneal fluid. In men the volume of peritoneal fluid is rarely greater than 5 ml in normal circumstances whereas in women the volume varies with the menstrual cycle and is typically between 5ml and 18 ml (Dhont et al., 1984). The pH of peritoneal fluid ranges between 7.5-8.0 and has significant buffering capacity. It consists mainly of water, electrolytes, plasma proteins such as fibrinogen and other solutes derived from interstitial fluid in adjacent tissues and from plasma of the supplying blood vessels due to the hydrostatic pressure gradient between plasma and the peritoneal compartment. Under normal conditions peritoneal cavity also contains various types of cells (Haney, 2000), varying in number, structure and type following disease, hence its diagnostic importance. The majority of cells are mesothelial cells, peritoneal macrophages, mast cells, fibroblasts and lymphocytes. Some particularly macrophages migrate freely between the peritoneal cavity and the surrounding tissue.

The amount of peritoneal fluid and plasma, and the levels of fibrinogen is greatly increased in the peritoneal cavity during post-surgical repair or following an inflammatory insult. Pathologically a high volume of peritoneal fluid is called ascitis. It occurs when venous outflow from the abdominal viscera is mechanically restricted, the

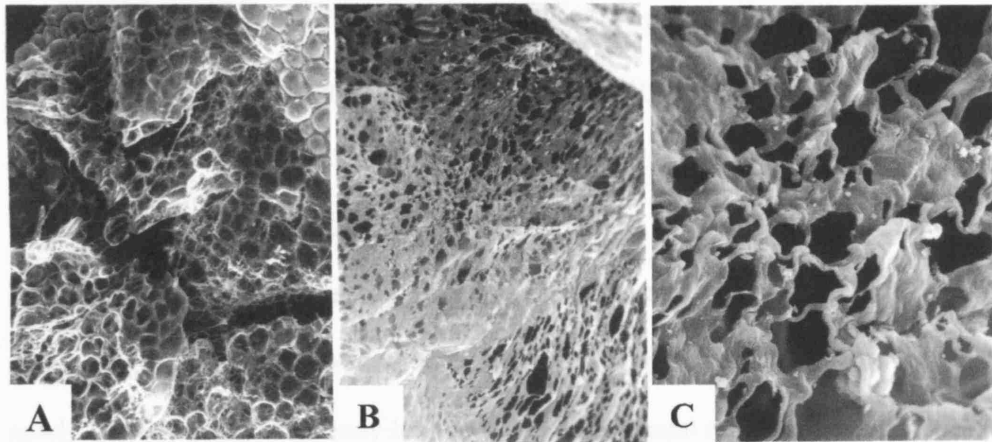
ovaries are therapeutically hyper-stimulated, venous outflow of the portal system is reduced due to cirrhosis or when there is an inflammatory process in the peritoneal cavity, such as endometriosis, tuberculoses or advanced cancer with extensive metastasis throughout the peritoneum. Clinically ascites is recognized when the amount of fluid present in the peritoneal cavity exceeds 1500 ml (Thompson, 2000, b).

A variety of cytokines such as interleukins, tumor necrosis factor (TNF) and interferon; enzymes (lysozyme, lipid peroxides); extracellular matrix proteins (glycosaminoglycans, fibrinogen, hyaluronic acid and fibronectin); growth factors (Macrophage colony-stimulating factor, vascular endothelial growth factor, macrophage-derived growth factor and transforming growth factor-beta; and other molecules such as proteases and inhibitors and carcino-antigens-125 (CA-125),  $\alpha_2$  – macroglobulin have been observed in the peritoneal fluid under varying clinical circumstances (Punnonen et al., 1996; Kligman et al., 1996; Armugam and Dip, 1995; Weinberg et al., 1991; Fakih et al., 1987; Olive et al., 1987). Although these factors are not unique to the peritoneal fluid their concentrations may be higher than in plasma if they are secreted by resident peritoneal macrophages or mesothelial cells leading to a concentration gradient from the peritoneal fluid to the plasma (Eisermann and Collin, 1989; Hoffman et al., 1988).

#### **1.5.8 The Omentum**

The omentum (Figure 1.5 A) always referred to as “policeman of the abdomen”, plays a role in protecting the peritoneal cavity from infection or irritative stimulants by adhering to sites of inflammation (Hall et al, 1998). During embryonic and foetal development the greater omentum forms from an expanded sacculation of the dorsal mesentery of the stomach and its surfaces fuse with each other to cover the anterior aspect of the intestine. It is the largest of the peritoneal folds, a double sheet, folded on itself to make four layers. The omentum is described as composed of 2 distinct types of

tissue, one adipose-rich which in the obese may be extensive, and the other is translucent and membranous containing numerous fenestrations (Wilkosz et al., 2005). Mesothelial cells form an almost continuous lining on both sides of the omentum and mesentery (Figure 1.5 B and C).



**Figure 1.5** (A) Scanning electron micrograph of the omentum of a mouse (x150). (B) Mesentery (x150) (C) Higher magnification of B (x600). Note the fenestrations in both structures.

Between the two layers of the anterior fold of the omentum close to the greater curvature of the stomach the right and left gastro-epiploic vessels form a wide anastomotic arcade. The omentum is the only site other than the diaphragm that has an ability to absorb particles more than 20,000 kDa from the peritoneal cavity. This is due to the presence of dense, oval or round aggregates of cells termed “milky spots” by the French anatomist Ranvier, 1874 (Vanvugt, 1996). They consist mainly of macrophages and lymphocytes surrounding profuse and characteristic capillary convolutions (omental glomeruli) that lie directly beneath the mesothelium (Wiffjels, 1992). The cells within the milky spots are supported by a delicate network of reticular fibres (Beelen, 1980) and are infiltrated by nerve fibres suggesting that milky spots are sites of possible immune-neuro-endocrine interactions (Krist et al., 1994). The number of milky spots is highest in infancy and gradually decreases with age, only becoming prominent in adults during intra-peritoneal infection. Migration of macrophages to and from the milky spots

into the peritoneal cavity is facilitated by the absence of elastic or basement membrane in the submesothelial connective tissue (Cranshaw & Leak, 1990). Following injury the omentum adheres to sites of inflammation, sealing them from the rest of the peritoneal cavity thus minimising systemic effects. The omentum also plays a role in the healing process either by providing an essential blood supply to ischaemic tissue or mounting an immunological response within the peritoneal cavity (Hall et al., 1996; Ellis, 1990; Myllareimi, 1967).

## **1.6 PERITONEAL HEALING**

During peritoneal healing, trauma initiates a cascade of events starting with an inflammatory phase and concluding when recruited cells have repaired the defect with newly formed tissue. This may lead to normal peritoneal repair or adhesion formation. The mechanisms regulating these two processes are unclear but are thought to overlap (Risberg, 1997). John Hunter in 1780 was one of the first to study the pathogenesis of adhesion formation and noted the early formation of fibrinous peritoneal deposits following inflammation. This was later identified as fibrin following the development of specific histologic staining techniques (Al-Musawi and Thompson, 2001). Several studies (Ellis et al., 1965; Milligan and Raftery, 1974) have since suggested that peritoneal healing is similar to cutaneous healing in that it follows three main sequences of events: inflammation, granulation tissue formation and tissue remodeling. However, there are obvious differences, especially in terms of re-epithelialization of the peritoneal defects by mesothelial cells and the presence of peritoneal fluid bathing the damaged serosal surfaces. The sequence of events known to be involved in cutaneous healing (reviewed by Clark, 1996) and thought to be in part involved in normal peritoneal repair will be discussed in the following sections.

### 1.6.1 Inflammation

Trauma to the peritoneum by surgical intervention, infection, radiation, thermal injuries or foreign bodies (such as reactive sutures, gloves talc powder and abdominal packs) usually causes the removal of the mesothelial layer. An inflammatory response leads to the expression and release of cytokines, arachidonic acid metabolites, oxygen-derived free radicals and growth factors. Damage to blood vessels and lymphatics result in extravasations of plasma and cellular elements such as platelets, erythrocytes and inflammatory cells. This initiates blood coagulation, vasoconstriction, and platelet aggregation to reduce blood loss followed by the formation of a fibrin-rich clot within 3 hours of injury to the peritoneum. The procoagulant thrombin is the activation product of prothrombin and has a central bioregulatory role in thrombosis/haemostasis in various disease states and healing. Thrombin converts soluble fibrinogen into an insoluble gel composed of polymerised fibrin monomers. Thrombin also binds avidly to fibrin and thereby proteolytically activates platelets and cleaves further fibrinogen. When activated platelets discharge their alpha-granules releasing several other proteins including fibronectin, thrombospondin and von Willebrand factor. In addition they release a variety of growth factors such as platelet-derived growth factor (PDGF), transforming growth factor-  $\alpha$  (TGF- $\alpha$ ) and transforming growth factor- $\beta$  (TGF- $\beta$ ) which promote subsequent granulation tissue formation.

The fibrin clot provides a provisional matrix in cutaneous wounds (Clark et al., 1982) and consists of additional extracellular molecules such as vitronectin and fibronectin (initially derived from platelets and plasma) and thrombospondin (derived from platelets). It also binds growth factors, proteases and their inhibitors. Through the cross-linking action of factor XIII it provides the substrate for the next phase of repair beginning within the first few hours of surgery. Leucocytes migrate to the peritoneal defect site in an orderly and timely sequence. The earliest cells to appear on the

damaged peritoneum are predominantly polymorphonuclear neutrophils (PMNL) that persist in large numbers for 1 to 2 days and then rapidly disappear if there is no infection. Neutrophils release proteases such as neutrophil elastase and collagenase that facilitate further cell extravasations from blood vessels. Neutrophils also destroy contaminating bacteria and foreign substances via phagocytosis and subsequent enzymatic and oxygen free radical mechanisms. Neutrophils play a role in wound healing by expressing cytokines and growth factors. Various chemotactic factors are released and attract further cells such as monocytes, lymphocytes and fibroblasts to the site of injury. These chemoattractants include fibrinogen cleavage products, fibrin degradation products, C5a arising from activated classical or alternative complement pathways, leukotriene B<sub>4</sub> released by activated neutrophils as well as a number of growth factors released by platelets.

The normal peritoneal cavity contains about 300 cells/mm<sup>3</sup> of which more than 85% are macrophages and the remainder mainly lymphocytes and desquamated peritoneal mesothelial cells (Haney, 2000). An inflammatory response to trauma or infection can result in a neutrophil count exceeding 3000 cells/mm<sup>3</sup> (Haney et al., 1981). One hour after trauma most of the resident macrophages have disappeared from the peritoneal cell population (Melnicoff et al., 1989) and are replaced by an influx of neutrophils and blood monocytes. Monocytes accumulation at the wound site continues for several days, stimulated by selective monocyte chemoattractants such as fragments of collagen, elastin, fibronectin and TGF-β<sub>1</sub>. Monocytes convert to macrophages and debride damaged tissues and pathogenic organisms by phagocytosis. They also release proteases such as collagenase and chemotactic factors that recruit additional inflammatory cells.

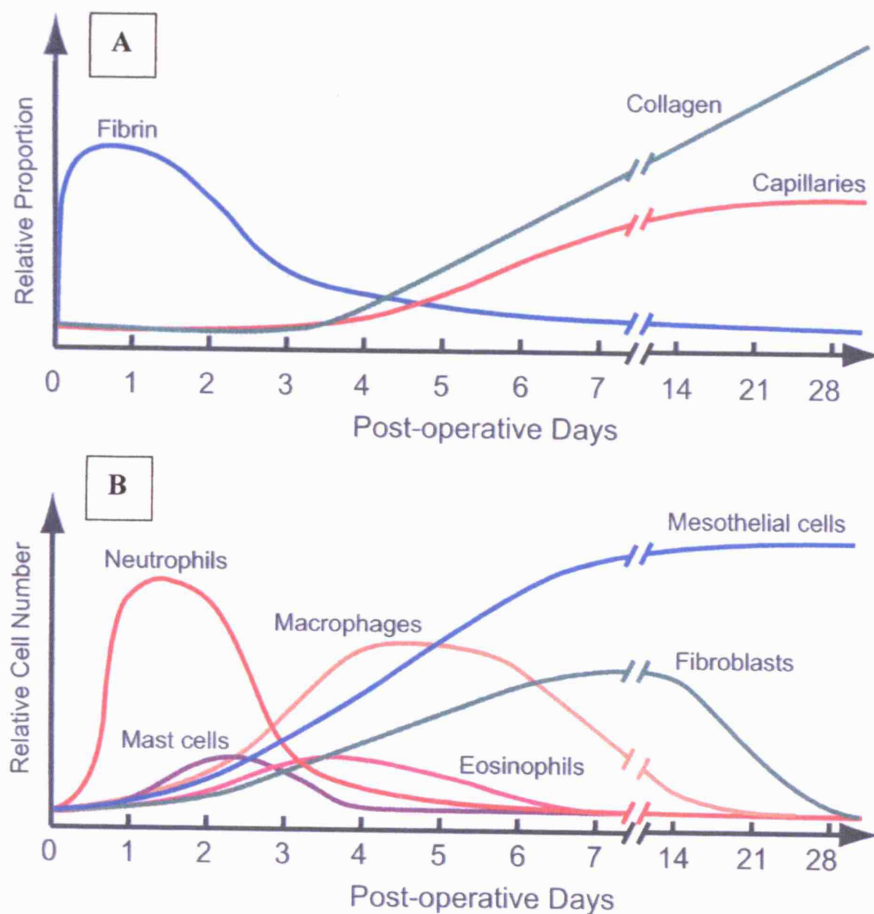
Peritoneal macrophages present on the surface of peritoneal injury site throughout the healing period play a pivotal role in the transition between inflammation



and repair (Haney, 2000). Macrophage-derived growth factors such as platelet-derived growth factor (PDGF), basic- fibroblast growth factor (b-FGF), transforming growth factor- alpha and beta (TGF- $\alpha$ , TGF- $\beta$ ) and epidermal growth factor (EGF), are necessary for initiation and propagation of granulation tissue formation in wounds since macrophage depleted animals have been shown to display defective cutaneous wound repair (Leibovich and Ross, 1975). After performing their acute inflammatory and scavenging roles they emigrate to the draining lymph nodes where they play an important part in the presentation of antigens from the inflamed site (Bellingan et al., 1996).

### **1.6.2 Granulation Tissue Formation**

As acute inflammation resolves granulation tissue begins to form approximately 3 to 4 days after injury and gradually replaces the fibrin clot (Figure 1.6). Cytokines and growth factors produced during the inflammatory phase act as chemoattractants, mitogens and regulate extra-cellular matrix (ECM) synthesis, and are therefore essential for granulation tissue induction in cutaneous wound healing. The provisional matrix promotes granulation tissue formation by providing scaffolding for contact guidance (fibronectin and vitronectin), low impedance for cell mobility (hyaluronic acid), and a reservoir of cytokines (Nathan and Sporn, 1991). It also directs signals to the cells through integrin receptors, so altering cellular function (Damsky and Werb, 1992). Significant numbers of fibroblasts appear in the avascular wound space by a combined process of migration and proliferation in situ termed fibroplasia. Fibroblasts migrate along concentration gradients of factors such as hypoxia, pH, growth factors, and other chemotactic factors. Fibroblasts within the cutaneous wound site gradually switch their major function to protein synthesis (Welch et al., 1990), and begin to deposit loose extracellular matrix composed of great quantities of fibronectin and collagen (Grinnell et al., 1981).



**Figure 1.6:** Changes in the relative number of blood vessels, fibrin, and collagen deposited (A) and cell types (B) at the site of peritoneal injury during the course of re-epithelialization. (Modified from Bridges & Whitting, 1964; Eskeland and Kjerheim, 1966; Raftery, 1973; Di Zerega, 1990).

Transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) induces fibroblasts to produce large amounts of collagen (Figure 1.7), initially collagen type I, and hyaluronic acid, and then collagen type III and sulphated glycosaminoglycans (GAG) (Ignatz and Massague, 1986; Roberts et al., 1986). Once abundant collagen matrix has been deposited in the cutaneous wound, fibroblasts cease collagen production despite the continuing expression of TGF- $\beta$ 1 (Clark et al., 1995). New blood vessel formation or angiogenesis

accompanies fibroplasia. Endothelial cells respond to angiogenic stimuli and migrate around the



**Figure 1.7:** Transmission electron micrograph of bundles of collagen fibrils in dense adhesion tissue in a mouse. The fibrils are sectioned transversely and longitudinally, the latter shows periodic cross-banding (x17000).

second day post-trauma by projecting pseudopodia through fragmented basement membrane. Subsequently endothelial cells migrate into the perivascular space while others remaining in the parent vessel proliferate on the second or the third day post-injury providing a continuing source of endothelial cells for the angiogenic process. Many factors are known to have angiogenic activity including basic fibroblasts growth factor (b-FGF), TGF- $\alpha$ , TGF- $\beta$ 1, tumour necrosis factor-  $\alpha$  (TNF- $\alpha$ ), platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), as well as low oxygen tension and lactic acid (Folkman and Klagsbrun, 1987).

Neuronal ingrowth follows a similar pattern to angiogenesis. Initially, growth factors released from inflammatory cells stimulate the growth of neuronal sprouts by activation of other related factors such a nerve growth factor (NGF).

### **1.6.3 Re-epithelialisation**

Hertzler (1919) found that both large and small peritoneal wounds healed within the same amount of time suggesting that peritoneal healing differed from that of skin.

Repair of the mesothelial layer begins within 2 to 3 days of peritoneal injury and there appear to be no difference in the nature of healing between peritoneal wounds at different sites (Ellis et al., 1962). However there is a difference in rate of healing of the visceral when compared with the parietal peritoneum as the visceral peritoneum (particularly the liver capsule) heals at a faster rate (Raftery et al., 1973). Korell et al. (1994) noted that for unknown reasons the inflammatory response to trauma of visceral peritoneum covering the ovary, fallopian tubes, uterus and bowel is more than the parietal peritoneum (pelvic or abdominal wall) response.

In the initial 48 hours the peritoneal surface is covered by macrophages embedded in the fibrin scaffold (Rodgers and Di Zerega, 1992). Over the next 2 to 5 days re-epithelialisation of the injured peritoneum occurs with a concomitant decrease in the macrophage population (Montz et al., 1987). The origin of the new mesothelial cells is controversial. The mesothelial basement membrane is destroyed in most wounds and mesothelial cells from the margins of the wound migrate over the provisional matrix and divide. However this process is not thought to be the major mechanism of repair. New mesothelial cells may also originate from primitive mesenchymal stem cells (MSCs) and/or subperitoneal fibroblasts (Ellis et al., 1965; Raftery, 1973). Lucas and colleagues (1996) investigated the role played by MSCs in adhesion formation and they observed that the introduction of viable MSCs immediately after surgery significantly reduced post-operative adhesions in rats. They suggested that MSCs have the capacity to differentiate into mesothelial cells capable of covering the injured mesothelium. In addition, it has been proposed that mesothelial cells detach from adjacent intact peritoneum and circulate in the serosal fluid (Foley-Comer et al., 2002), then re-attach to the injured site, proliferate and form a continuous sheet of mesothelial cells (Cameron et al., 1957, Bridges and Whitting, 1964; Mutsaers et al., 2000). Once re-epithelialisation is completed usually by days 5-8 (Eskeland et al., 1966; Hubbard et al.,

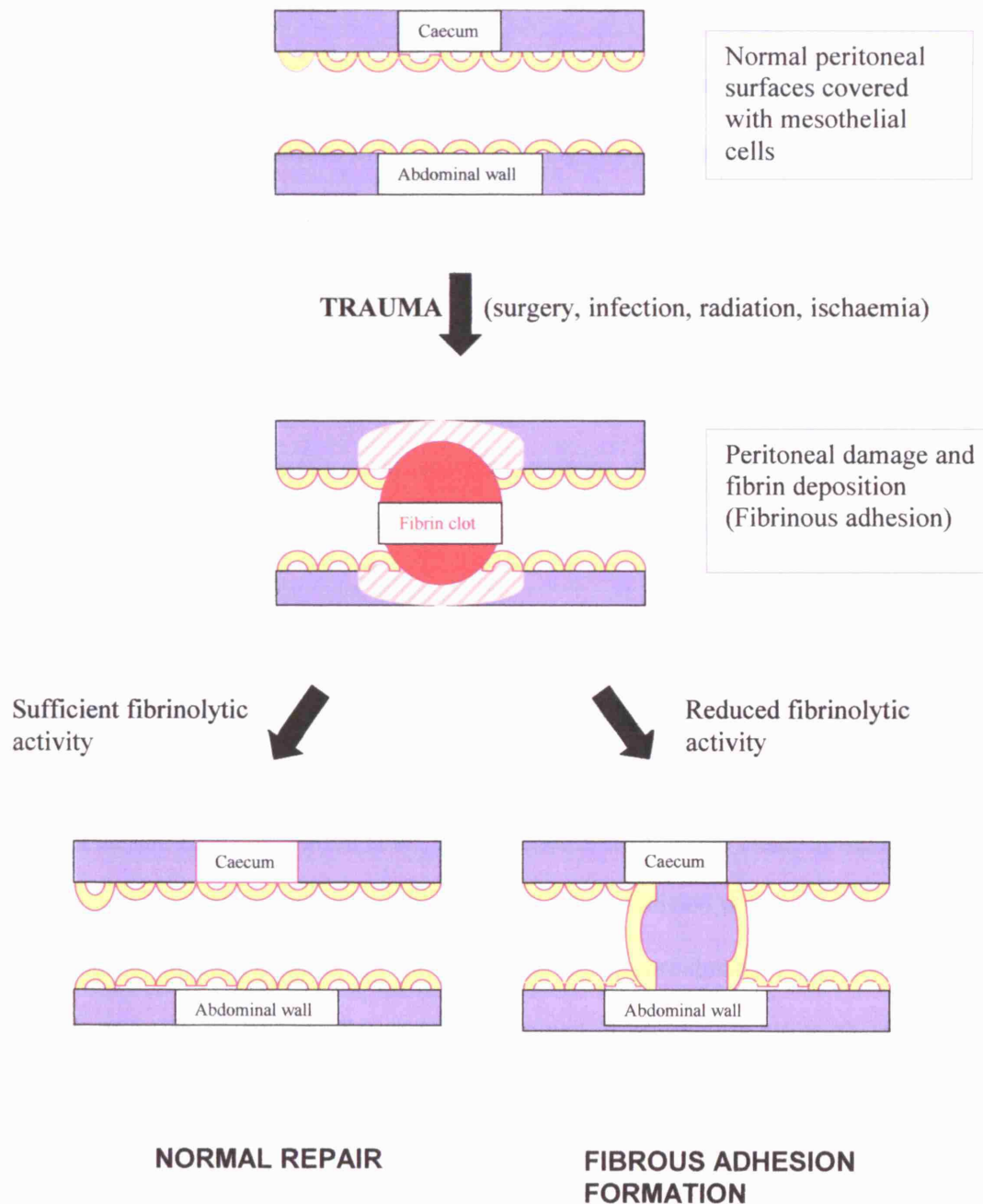
1967) the basement membrane reforms and the cells bind firmly to each other and to the basement membrane via new adhesion molecules such as desmosomes and hemidesmosomes (Foley-Comer et al., 2002)

#### **1.6.4 Tissue Remodelling**

This is considered as the last phase of wound repair where cell apoptosis or programmed cell death takes place. Endothelial cells appear to be the first cell type to undergo apoptosis followed by fibroblasts leading to a less vascular and cellular scar tissue. In the later months remodelling of extracellular matrix (ECM) occurs as a continuous albeit slow process (Compton et al., 1989). Fibronectin and hyaluronic acid decrease and collagen bundles grow in size. The stability of the fibres increases progressively with the formation of covalent cross-links between adjacent molecules increasing wound tensile strength. Myofibroblasts cause contraction of cutaneous wounds (reviewed by Clark 1996). However the same process is not known to occur in peritoneal healing.

### **1.7 MECHANISM OF ADHESION FORMATION**

While few studies based on animal models have investigated the mechanisms of peritoneal adhesion formation, it is generally agreed that they develop from a fibrinous provisional matrix between opposing damaged surfaces (Figure 1.8), leading to a number of evolving theories addressing their pathogenesis, each targeting one aspect of a multi-faceted process. The main factors involved in adhesion formation are thought to be close apposition of damaged surfaces and reduced fibrinolytic activity (Figure 1.8). A variety of animal models will be discussed briefly in the following section and then the role played by each of the initiating factors such as trauma or surgical intervention, infection, ischaemia and foreign bodies in adhesion formation, followed by the role of coagulation, fibrinolysis, inflammation and release of cytokines/ growth factors.



**Figure 1.8:** Hypothesis of normal peritoneal tissue repair and the effect of the fibrinolytic pathway in adhesion formation (Sulaiman et al., 2002).

### 1.7.1 Animal Models

Animal models have been used for scientific research to elucidate a concept or test a hypothesis. Various approaches in the understanding and prevention of adhesions have been gained by studies on animal models. Many research groups have designed a variety of experimental models using different species (mice, rats, rabbits, monkeys, dogs, pigs and horses) depending on the area of interest. In order to assess precisely post-operative peritoneal adhesions it is essential to have a standardized and reproducible model to allow a quantitative assessment. As the pathogenesis of intra-peritoneal adhesions is multi-factorial, different models are employed and are briefly described.

(i) *Surgical trauma model*: has been used extensively with a variety of approaches depending on the severity of trauma required. Through a midline abdominal incision, trauma is applied to the abdominal/pelvic wall and the adjacent colon (Rodgers et al., 1997) or uterine horn (Nishimura et al., 1984; Ricketts et al., 1999) either by abrasion (using gauze, scalpel or brush), pressure, dissection or excision resulting in bleeding. The wound is stretched or damaged and serosal surfaces approximated with sutures.

(ii) *Ischaemic model*: the blood supply to an organ is interrupted by crush injuries, ligation, or by stripping, excision or compression (Buckman et al., 1976; Wiseman et al., 1994; Burns et al., 1997).

(iii) *Infection model*: the peritoneal cavity is infected by soiling with faecal material or the introduction of bacteria (O'Leary et al., 1988). Brolin (1984) fed the rats with a special diet that changed the intestinal microflora resulting in peritoneal sepsis and abscesses followed by adhesion formation.

(iv) *Foreign body model*: starch, talc powder, gauze, mesh or suture materials are introduced into the peritoneal cavity (Baykal et al., 1997; Farmer et al., 1998). Bakkum

et al. (1995) created oval shaped peritoneal defects and stitched them with different suture materials to assess adhesion formation.

There is still no standard adhesion animal model available. An animal model must be consistent, reliable, and reproducible that can be compared with both positive and negative controls. It should not be either very severe that no agent reduces adhesion formation, or too permissive such that all agents are efficacious (Wiseman, 1994). The main objective of most adhesion animal models is to consolidate our understanding of patho-physiological mechanisms of adhesion formation in order to identify preventive or therapeutic measures.

### **1.7.2 Histology of adhesion formation**

Few studies have addressed the events involved in adhesion formation by performing a histological time course. Milligan and Raftery (1974) described the histological features of adhesion formation in rats using light and transmission electron microscopy. They compared adhesions arising from the liver with those from the caecum and ileo-cecal junction using both peritoneal abrasion and stripping techniques to create petechial bleeding or a clear, well-demarcated peritoneal defect. Rats were killed at 1-7 days and 2, 4 or 8 weeks after the initial trauma. At days 1 to 3 the adhesion was characterized by a variety of cellular elements embedded in a fibrin matrix. The cells were primarily neutrophils but also included macrophages, eosinophils, red blood cells, and tissue debris as well as necrotic cells. By 4 days macrophages were the predominant leukocyte in the fibrin mesh, which primarily contained large strands of fibrin associated with fibroblasts. A few mast cells were seen at day 5 with many fibroblasts found together with macrophages assuming the formation of a syncytium. Distinct bundles of collagen fibrils were evident and in several sites were encased with scattered foreign-body granulomas. At 7 days collagen and fibroblasts were the main constituents of the adhesion. However small vascular



channels lined with endothelial cells were present. The number of mast cells increased between 2 weeks and 2 months. During this time the cellularity of adhesion had decreased and was replaced mainly by collagen fibrils and fibroblasts.

### **1.7.3 Role of trauma/laparoscopy on adhesion formation**

Under optimal conditions and with the most meticulous technique, surgical procedures still damage the peritoneum and with the close opposition of traumatized peritoneal surfaces result in adhesion formation (Lamont et al., 1992; Haney 1994). Other events are also important such as drying of the serosa and thermal injuries (Ryan et al., 1971), as well as different types of surgical gauze and suture materials result in different types of peritoneal damage and subsequent adhesion formation (Tol et al., 1997).

It has long been assumed that fewer adhesions are induced by laparoscopy than by laparotomy (Maier et al., 1992) but there is lack of documented evidence. In general, the two major pre-requisites to adhesion formation are tissue drying and peritoneal damage and these occur during insufflation of raw gas into the abdomen during laparoscopy. Histological evaluation of peritoneum exposed to direct gas flow from laparoscopy showed that mesothelial cells were removed from the peritoneal surface. The longer the procedure or the larger the volume of gas insufflated the greater was the loss of peritoneal continuity and integrity (Molinas et al., 2003). This effect may be a factor in “*de novo adhesion formation*” (the occurrence of adhesion at non-operated sites).

Jenson and colleagues (1992) performed a second-look laparoscopy 12 days after performing 256 consecutive operations for infertility for early treatment of post-operative adhesions. Adhesions were present in half the patients treated by either previous microsurgical technique or laparotomy (open technique). Whereas “*adhesion reformation*” (the recurrence of adhesions at the operated site) occurred in 90% of those

cases, but in laparoscopic surgery there were fewer “de novo adhesion formation”. They concluded that adhesion reformation is frequent and depends on the initial extent of surgery. In a prospective randomized study by Lundorff et al. (1991), 105 patients with ectopic tubal pregnancy were randomized to laparoscopy or laparotomy. Seventy-three patients underwent a second-look laparoscopy to evaluate adhesion formation and tubal status. Adhesions were found in 58% of patients who were adhesion free at the initial laparoscopy and in 79% after laparotomy. Among the patients who were found to have adhesions at the first procedure, the adhesion status was slightly impaired after laparoscopy, but developed dramatically after laparotomy, on the ipsilateral tube as well as on the contralateral side. From this study, they concluded that laparoscopic surgery induces less de novo adhesion formation and reduces the risk of adhesion formation.

Although evidence suggests that laparoscopy reduces the risk of de novo adhesion formation, adhesions are as frequent as after laparotomy. It has been recommended that patients with previous laparotomy should undergo a microlaparoscopy under direct vision before having further laparoscopic abdominal surgery in order to prevent any visceral injury (Audebert and Gomel, 2000).

#### **1.7.4 Role of ischaemia in adhesion formation**

Ischaemia caused by vascular ligation, devascularization of tissues by stripping, circumferential suturing and cautery induces injury that may result in tissue death and adhesion formation. Assessment of the effect of ischaemia was performed by comparing two identical rabbit models. One model devascularization and abrasion of the uterine horn was performed resulting in adhesions formation between the uterus and other organs remote from the primary injury (Wiseman et al., 1994). The other by abrasion alone leading to adhesion formation restricted to the uterus. This suggested that ischaemia results in the release of mediators able to induce injury at secondary sites (Wiseman et al., 1992).

It has been proposed that degradation of fibrinous exudate depended on an intact mesothelium which if damaged adhesions developed (Haney, 2000). However it is now clear that large peritoneal defects can heal without adhesion formation unless the wound edges were opposed with sutures. Ellis (1962) concluded that it was not the peritoneal defect that stimulated adhesion, but the presence of ischaemic tissue that probably resulted from pulling the wound edges under tension. Hubbard and co-workers (1967) noted that closure of a laparotomy wound without suturing the peritoneum might decrease the incidence of adhesion formation. Booth and colleagues (1973) showed in an ischaemic model associated with strangulating obstruction of the small intestine in dogs that there was partial loss of the mesothelium in the first hour that was complete at 6 hours. Raftery (1973) suggested that ischemia might prevent proliferation of submesothelial cells, leading to a delayed conversion into mesothelial cells.

It therefore appears ischemia causes loss of mesothelium leading to adhesion formation and that regeneration of the mesothelium without adhesion formation depends on the rapid invasion of fibrinous exudates by mesothelial cells and on their fibrinolytic activity.

Some studies suggest that adhesions form only following anoxic peritoneal defects, where they act as vascular grafts from one organ to another to prevent necrosis of anoxic tissue (Ellis, 1962; Glucksman, 1966; Lundin, 1989). Myllarniemi and colleagues (1968) supported this concept by showing that the regeneration of new blood vessels from omental adhesions began as early as 24 hours after surgery. In another study omentectomy resulted in gangrene of the organ or tissue necrosis as it was deprived of its blood supply (Ehrler, 1966). The omentum is particularly susceptible to adhesion formation following trauma as shown in a post-mortem study where omental adhesions were found in 92% of patients with post-operative adhesions (Weibel and

Majno, 1973). Kuebelbeck et al. (1998) also found that omentectomy reduced post-operative adhesions in horses.

#### **1.7.5 Role of infection in adhesion formation**

Intra-peritoneal infection independent of a particulate or chemical irritant is a potent promoter of adhesions although the mechanisms involved have not been clearly established. Bacteria cause direct serosal damage and activate various cascade systems including the coagulation cascade by the release of endotoxins (Chapman et al., 1983). Bacteria secrete vasoconstrictors and chemoattractants for inflammatory cells (Holtz et al., 1984). In peritoneal infection there is massive influx of leukocytes (predominantly neutrophils, but also significant numbers of mononuclear cells) into the peritoneal cavity. Data from studies examining peritoneal effluent following peritonitis and stable peritoneal dialysis suggest that both peritoneal macrophages and mesothelial cell products potentially contribute to peritoneal inflammation (Goldman et al., 1990; Bagby et al., 1991). The initial view on peritoneal host defense suggested that peritoneal macrophages controlled the peritoneal response to bacterial invasion. However observations made with cultured human peritoneal (and pleural) mesothelial cells suggest that many of the inflammatory mediators locally produced within the peritoneal cavity during inflammation are potentially of mesothelial cell origin and play a central role in initiating, amplifying and controlling peritoneal inflammation (Topley and Williams, 1994). Activation of the mesothelium by peritoneal macrophages-derived pro-inflammatory cytokines (IL-1 $\beta$  and TNF- $\alpha$ ) is thought to play a role in this process (Fieren et al., 1990).

Bacterial and faecal contamination of the peritoneal cavity that commonly occurs in bowel surgery is a potent stimulus of adhesiogenesis (O'Leary et al., 1988). Surgical trauma as well as peritonitis, depresses peritoneal fibrinolytic activity (van Goor et al., 1994), and so consequently increase adhesion formation. This was

confirmed in rats undergoing laparotomy after intra-peritoneal inoculation with pure bacterial cultures or saline (O'Leary and Coakley, 1992). In animals that had saline inoculums and the peritoneum sutured with nylon adhesions to the laparotomy scar was observed in 3 out of 10 animals. While with intra-peritoneal infection adhesion to the laparotomy scar occurred in 8 out of 9 animals when the peritoneum was sutured with nylon, but only in 2 out of 10 when left unsutured. Bothin et al. (2001), found that bacterial flora of the gastrointestinal tract enhanced adhesion formation around surgical anastomoses. They found that the adhesion response in germ-free rats was significantly lower compared with conventional ex-germ-free and *E. coli* contaminated rats. It was suggested that bacterial translocation or simple leakage or migration along sutures or through small deficiencies in the anastomosis might stimulate adhesion formation. In these circumstances adhesions are beneficial as they seal off foci of infection (Vance and Williams, 1972; Ellis, 1980; Fabri et al., 1983)

#### **1.7.6 Role of foreign bodies in adhesion formation**

The association of foreign bodies such as surgical gauze, talc, starch or other particulate from glove powder, lint from drapes, packs or gowns and suture materials with adhesion formation is well recognized (Holmdahl and Risberg, 1993; Ellis, 1994; Bakkum et al., 1995). Adhesions are frequently observed to form on or to extend from sutures and ligatures as they evoke an inflammatory reaction (Schade and Williamson, 1968; Riddick, 1977). The suture material is more central in inducing adhesions than its diameter, size or knot configuration (Hubbard, 1967; Bakkum, 1994). Another study found starch granulomas in 25% of patients who had undergone prior operations by surgeons wearing starch-containing gloves (Veress et al., 1991; Luijendijk, 1996). Of clinical significance starch contamination can induce peritonitis (Hugh et al., 1975; Ellis, 1990) associated with bowel obstruction, ileus, fever and leucocytosis, sometimes termed “the starch peritonitis syndrome” (Klink and Boynton, 1990).

Schade and Williamson (1968) evaluated the temporal course of adhesion formation in rats after the addition of foreign body (colloidal silica oxide) into the peritoneal cavity. Three phases of adhesion formation were noted by light and ultrastructural assessment. The first phase (0-7 hours) involved degeneration and desquamation of mesothelial cells. In the second phase (7 hours-10 days), fibrin deposition on exposed basement membrane led to the formation of fibrinous adhesions. The transformation of fibrinous exudates into fibrous adhesions occurred over an extended period of time (10 days- 1 month).

It is obvious that preventive measures can reduce or prevent the occurrence of post-operative adhesions. Starch-containing gloves have been banned in the UK and many other countries. Using fine instruments, meticulous techniques and infection prophylaxis are important.

#### **1.7.7 Role of inflammation in adhesion formation**

Inflammation is known to be involved in adhesion formation and a number of studies have suggested that specific inflammatory cells play a key role. Inhibition of neutrophil-endothelial cell interactions by monoclonal antibodies directed against cell receptor CD11a/CD18 was shown to prevent neutrophil activation and migration and consequently increased adhesions following a surgical injury (Ar'Rajab et al., 1996). This was thought to be due to lack of neutrophil proteinases such as matrix metalloproteinases and serine proteinases that degrade extracellular matrix and tissue debris. Haney (2000) noted that peritoneal macrophages were present at the peritoneal injury site throughout the healing period and Fukasawa and co-workers (1987) reported that activated macrophages facilitate fibroblast proliferation and protein secretion. However during adhesion formation enhancement of peritoneal macrophages reduced markedly the degree of post-operative adhesion formation (Ar'rajab et al., 1995).

Mast cells are thought to be involved in adhesion formation following peritoneal inflammation resulting from either immunologic stimulation or chemical irritation (Gotloib et al., 1995). They produce a number of inflammatory mediators including histamine, serotonin, platelet activating factor, eicosanoids, most prominently LTD<sub>4</sub> and prostaglandin D<sub>2</sub> (Schwartz, 1987). Mast cell degranulation and proliferation has been documented after simple handling of the intestine (Bos et al., 1991; Vermillion and Collins, 1993; Gotloib et al., 1996), and mast cell stabilizers such as disodium cromoglycate, nedocromil and 5HT<sub>2</sub> receptor blockade that affect degranulation, significantly attenuate adhesion formation in a dose-dependent fashion in a rat model (Leibman et al., 1993; Langer et al., 1995).

#### **1.7.8 Role of cytokines and growth factors in adhesions formation**

During peritoneal inflammation resident cells such as mesothelial, macrophages, fibroblasts and infiltrating leucocytes produce cytokines and other inflammatory mediators (Ivarsson et al., 1998). Several growth factors and cytokines are present in peritoneal fluid and their receptors are expressed on inflammatory cells at the site of injury suggesting a role in regulation of peritoneal wound healing (Overall, 1994; Chegini, 1994). Intra-peritoneal fluid levels of pro-inflammatory mediators, such as tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-1 & 6 (IL-1 and 6), levels increase during and after episodes of trauma or infection (Brauner et al., 1996; Tekstra et al., 1996). Furthermore post-operative administration of IL-1 was shown to increase adhesion formation in rats (Herschlag et al., 1991). Transforming growth factor- $\beta$  (TGF- $\beta$ ) is thought to play a key role in peritoneal healing and fibrous adhesion formation as it is chemotactic for fibroblasts and inflammatory cells and promotes cell proliferation, differentiation and angiogenesis as well as regulating the expression of various components of the extracellular matrix (Cormack et al., 1987; Williams et al., 1992; Ketteler et al., 1994; Bordes and Noble, 1994)).

Vascular permeability factor (VPF)/vascular endothelial growth factor (VEGF) is also thought to contribute to the development of adhesions and blocking an antibody to VEGF lead to less adhesion formation (Saltzman et al., 1996). Vascular endothelial growth factor is secreted by a variety of normal and transformed cell types and is 1000 times more potent on a molar basis than histamine in promoting vascular leakage. It has been shown to contribute to the vascular hyperpermeability of peritoneal tissue and to the subsequent deposition of fibrin after injury (Neufeld, 1994).

#### **1.7.9 Role of coagulation in adhesion formation**

The role of blood in the formation of adhesions is controversial. Ryan et al. (1971) showed that addition of fresh blood or preformed clots intra-peritoneally to an otherwise uninjured peritoneal cavity in rats resulted in omental adhesion formation. When 0.2 to 2 mL of fresh blood was dripped onto a dried peritoneal surface and allowed to clot adhesions formed at the site of dried surface. When the peritoneum was also injured by excision adhesion formation was markedly enhanced. On the other hand clotted blood was completely absorbed by a normal peritoneum within 48 hours (Hertzler, 1919), whereas well formed clots were absorbed within 8 days (Jackson, 1958). Larsson (1997) suggested that trauma to the serosa rather than blood was the initiator of adhesion formation. Bronson and Wallach (1977) found that 46% of their infertile patients with pelvic adhesions had no predisposing factors, suggesting that bleeding with follicle rupture at the time of ovulation was responsible. Golan and Winston (1989) confirmed the same findings and reported that blood in conjunction with trauma to the serosa produce more adhesions than trauma alone or trauma plus serum. The role of fibrin in the formation of adhesions is suggested from the observation that use of defibrinated blood or blood products are less frequently associated with adhesion formation. Thrombin binds to fibrin and proteolytically cleaves fibrinogen. The interaction of thrombin with biologic substrates involves



various binding sites that are bound by hirudin, a potent natural inhibitor of thrombin (Kelly, 1992). A recombinant hirudin analog (rec-Hirudin) was shown to reduce adhesion formation through inhibition of thrombin (Rodgers et al., 1996).

#### **1.7.10 Role of fibrinolysis in adhesion formation**

The importance of fibrin deposition and the role of fibrinolysis in adhesion formation following peritoneal trauma is well recognized. In 1969, Myhre-Jensen and colleagues suggested that mesothelial cells activate the principal fibrinolytic protease plasminogen. Gervin and co-workers (1973) demonstrated fibrinolytic activity in all the gastrointestinal serosa with activity maximal in the mid- to distal ileum and proximal colon. These areas seem to have the greatest incidence of adhesion formation after surgery. They found that the fibrinolytic activator activity in abdominal tissue was of mesothelial origin and was significantly reduced by trauma. It is now well established that surgery affects the systemic fibrinolytic response and therefore changes in peritoneal plasminogen activity could be part of a generalized response (D'Angelo et al., 1985; Kluft, 1990; Rout & Diamond, 2003).

In summary, the critical factors in adhesion formation are thought to be the close apposition of serosal surfaces and whether fibrin is lysed or organized into fibrous tissue. This is dependant on equilibrium between fibrin deposition and fibrinolysis. When the amount of fibrin deposited is beyond the capacity of the peritoneal fibrinolytic activity the persistence of fibrin matrix allows the migration of fibroblasts and endothelial cells, resulting in the formation of fibrous adhesions. The importance of the fibrinolytic system in the formation or pathogenesis of post-operative adhesion formation will be discussed in more detail in the following section.

### **1.8 THE FIBRINOLYTIC SYSTEM**

The fibrinolytic system involves the lysis of fibrin into fibrin degradation products (FDPs) (Vassalli et al., 1991; Lijnen et al., 1994). The main component of the

fibrinolytic system is plasminogen, an inactive proenzyme which is converted to the active enzyme plasmin by two distinct plasminogen activators (PAs): the tissue type plasminogen activator (t-PA) and the urokinase-type plasminogen activator (u-PA) enzymes (Rijken et al., 1981; Collen and Lijnen, 1991; Blasi, 1993). Tissue-PA differs from u-PA in molecular weight, immunological specificity and kinetic properties and is derived from different genes. Through the generation of non-specific and potent protease plasmin, PAs regulate numerous important biological processes, including fibrinolysis, thrombolysis, cell migration, atherosclerosis, reproduction, embryogenesis, wound healing, invasiveness and metastasis (reviewed by Vassalli, 1994).

The PAs are produced and secreted from cells in the form of proenzymes, which are transformed to active enzymes by a specific proteolytic cleavage. Inhibition of the fibrinolytic system occurs either at the level of PA by specific plasminogen activator inhibitors (PAIs) (Schneiderman and Loskutoff, 1991) or at the level of plasmin mainly through inhibition by  $\alpha_2$ -antiplasmin (Figure 1.9).

### **1.8.1 Regulation of plasminogen activators/ plasmin system**

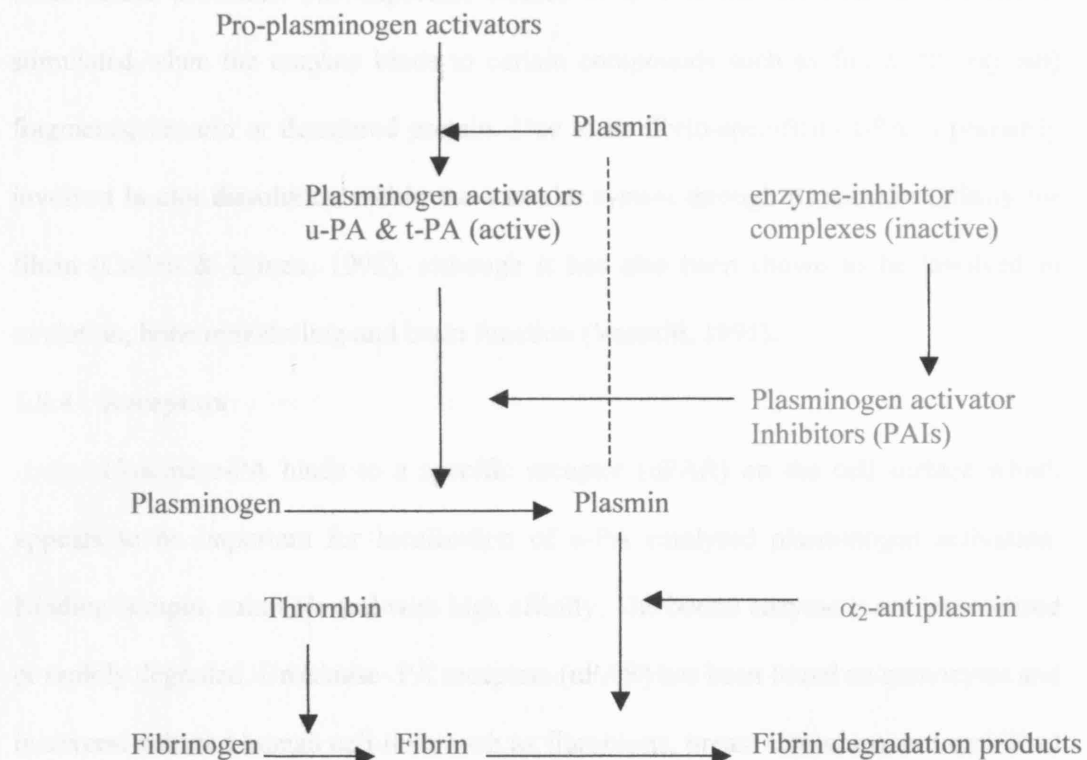
The range of cell types that can produce one (or more) of the constituents of the PA/plasmin system is extremely broad. Plasminogen activators and PAI-1 are normally present in blood plasma, liver hepatocytes, endothelial cells, monocytes, and PAI-2 during late pregnancy.

The intricate regulation of PA and PAI synthesis allows expression of the PA/plasmin system to be precisely controlled and is dependent on growth factor balance. One striking illustration of this is provided by the effect of basic-FGF and TGF- $\beta$ 1 on endothelial cells. Both agents enhance PAI-1 and uPA mRNA and protein synthesis, but the kinetics and amplitudes of the changes in expression of the two genes are remarkably different so that the overall balance is tilted towards proteolysis in response to b-FGF and inhibition of proteolysis in response to TGF- $\beta$ 1. It is generally

considered that PAs and PAIs follow the constitutive secretory pathway, in that secretion rapidly follows synthesis. However immunohistochemical analysis of u-PA in the mouse vas deferens suggests that the antigen is stored in apical granules and can therefore follow the pathway of regulated secretion. Also in humans PMNLs expression of u-PA activity at the cell surface can be triggered by exposure to various stimuli such as trauma or infection. The concentration of PAs varies greatly among different animal species being high in rats and less so in rabbits, and as well varies among different tissues being particularly high in vascularized connective tissues such as the liver (Myhre-Jensen et al., 1969). In healthy young individuals moderate exercise results in increased plasmin formation while heavy exercise causes the generation of plasmin to exceed that of thrombin and fibrin (Weiss et al., 1998).

#### **1.8.2 Urokinase - plasminogen activator (u- PA) and its proenzyme**

Single-chain urokinase-type plasminogen activator (scu-PA) is a 54-kDa glycoprotein molecule composed of two polypeptide chains of 24 K Da and 30 K Da linked by one disulphide bond. It is formed of several domains: the NH<sub>2</sub>-terminal chain is formed of an epidermal growth factor domain, one kringle region and the proteolytic region. The epidermal growth factor domain is responsible for the binding of the single-chain uPA to its receptor. The plasma concentration of scu-PA is about 2nmol/L. It is secreted by a variety of cells and is present in the extracellular fluid in the form of an inactive single chain proenzyme (pro- u PA) (Lijnen et al., 1994). Conversion of pro-u PA to active chain u-PA by catalytic amounts of plasmin is a critical regulatory step in plasminogen activation. By this mechanism initial traces of plasmin catalyse the production of active u-PA which leads to the formation of more plasmin. Through binding to its receptor (u-PAR), uPA functions in cell mediated proteolysis causing localized dissolution of extracellular matrix and cellular transversion of basement membrane in processes such as migration, ovulation, inflammation, tissue remodeling



**Figure 1.9:** The Plasminogen Activation Cascade

and metastasis (Dano et al., 1994). Pericellular proteolysis occurs either directly or by plasmin activation or via activation of other latent proteinases (He et al., 1989; Murphy and Reynolds, 1992, Vassalli, 1994).

### 1.8.3 Tissue-plasminogen activator (t-PA) and its proenzyme

Tissue type-PA is a 70 kDa glycoprotein. The NH<sub>2</sub>-terminal region is formed of several domains: finger domain, growth factor domain, two kringles and the proteinase domain. Its concentration in human plasma is 0.1 nmol/L and the half-life of t-PA in the circulation is about 5 minutes as a result of rapid hepatic clearance (Lijnen et al., 1994). The enzyme is synthesized as a single chain molecule (sc-tPA); its catalytic activity increases as a result of plasmin-mediated conversion to a two-chain molecule (tc-tPA). One chain is a heavy 38 kDa polypeptide bearing sites responsible for enzyme regulation and the other light 31 kDa chain contains the active site similar to that of

other serine proteases. An important feature of tPA is that its catalytic activity is stimulated when the enzyme binds to certain compounds such as fibrin, fibrin(ogen) fragments, heparin or denatured protein. Due to its fibrin-specificity t-PA is primarily involved in clot dissolution within the vascular system through its specific affinity for fibrin (Collen & Lijnen, 1995), although it has also been shown to be involved in ovulation, bone remodelling and brain function (Vassalli, 1991).

#### **1.8.4 Receptors**

Urokinase-PA binds to a specific receptor (uPAR) on the cell surface which appears to be important for localization of u-PA catalysed plasminogen activation. Binding is rapid, saturable and with high affinity. The bound enzyme is not internalised or rapidly degraded. Urokinase- PA receptors (uPAR) has been found on monocytes and in several cultured human cell lines such as fibroblasts, breast carcinoma and umbilical vein endothelial cells. Binding involves the "growth-factor like" domain of uPA and receptor-bound u-PA remains functionally active at the cell surface with a half-life of 4-5 hrs. Single chain pro-u PA binding to the cellular receptor is followed by conversion of the bound enzyme to active two chain u-PA. Receptor bound u-PA can bind PAI-1 maintaining its susceptibility to inhibition. Simultaneous binding of pro-uPA, uPA, plasminogen and plasmin at the cell surface produces a topographical proximity which is important for the biological function of the plasminogen activation process. The proximity enables efficient autocatalytic activation of plasminogen by u-PA and serves to localise the proteolytic activity. Thereby, the cells are armed with a matrix-degrading activity and the receptor controls the position of plasmin activity at focal contact sites. Cell invasiveness and cell migration depend on this focal localisation.

Various cells including endothelial, mesothelial, macrophages and fibroblasts also have receptors on their surfaces that bind the substrate, plasminogen and t-PA. Some receptors are clearly involved in the clearance (in particular by the liver) of the

enzyme or of tPA-PAI complexes whereas others localize t-PA to the plasma membrane.

### **1.8.5 Inhibitors**

Time and site specific inhibition of the catalytic activity of PAs is instrumental in the prevention of aberrant plasminogen activation and potentiate tissue damage. In the circulation PA inhibitors block premature fibrinolysis. Plasminogen activator inhibitor-1 (PAI-1) is the major inhibitor in plasma. It has high affinity for tPA, both single chain (sc) and two chains (tc), as well as for uPA. However, PAI-2 only inhibits uPA and also albeit less efficiently tPA (tc, but not sc). Plasma levels of PAI-1 and PAI-2 are elevated in conditions associated with enhanced tendency for thrombus formation (Schneidermann & Loskutoff, 1991). PAI-1 is secreted as an active anti-protease but it rapidly converts to an inactive latent form. Binding of t PA to fibrin reduces the ability of PAI-1 and PAI-2 to react with the enzyme. Other inhibitors that play a role in the regulatory mechanisms are  $\alpha_2$  – antiplasmin, the main plasmin inhibitor in plasma and the protease nexin that inhibits uPA, plasmin and thrombin activity.

### **1.8.6 Plasminogen activators and inhibitors in adhesion formation**

During the initial phase of peritoneal tissue repair (0-3 days post-injury) adhesions consist of a fibrin network infiltrated by inflammatory cells (Raftery, 1973). Early studies demonstrated that the peritoneal cavity possesses plasminogen-activating activity (PAA) (Porter et al., 1971) as well as inhibitors of fibrinolysis (Pugatch & Poole, 1968). Based on these observations it was demonstrated that reduction in peritoneal PAA due to surgical trauma was associated with increased adhesion formation in many animal models (Buckman et al., 1976). PAA was found to be significantly reduced intra- and post-operatively in humans, while PAI-1 and PAI-2 levels were increased (Vispand et al., 1990; Whawell et al., 1993; Holmdahl, 1997). PAI-1 and PAI-2 concentrations are increased during surgical procedures and in

inflamed peritoneum as in appendicitis associated with a reduction in peritoneal PAA (Scott-Coombes et al., 1995; Holmdahl et al., 1998). Fibrinolytic activity begins to rise 3 days after peritoneal trauma and increases to a maximum at day 8 (Pados et al., 1992) and lasts up to a month (Bakkum et al., 1996). However following colonic anastomosis, Reijnen et al. (2002) refuted an early increase in peritoneal t-PA levels followed by an increase in t-PA fibrinolytic activity. Ivarsson et al., (2001) found that t-PA is rapidly released by the visceral peritoneum during abdominal surgery in humans. They suggested that this was an active process due to different concentrations of t-PA in the serosal fluid from the small bowel and peripheral blood but there was no significant difference in u-PA and PAI-1 concentrations.

In cultured human mesothelial cell lines, PAI's were increased in the presence of bacterial lipopolysaccharide and inflammatory mediators such as IL-1 and tumour necrosis factor-alpha (TNF- $\alpha$ ). Such factors are present in the peritoneum following inflammation and so may indirectly inhibit lysis of fibrinous deposits within the abdominal cavity and promote adhesion formation (Whawell et al., 1994). A reduction in t-PA activity was observed in noninflamed as well as in inflamed peritoneum, indicating the effect caused by the operative trauma (Holmdahl, 1996).

With the recent availability of recombinant tissue plasminogen activator (rt-PA) studies have demonstrated that its administration into the peritoneal cavity in excess of the inhibitor activity corrects the deficit in fibrinolytic activity and reduces or eliminates adhesion formation (Menzies & Ellis, 1991). However this also produces a significant impairment of the early phase of wound healing. Braaten et al., 1997 observed ultrasonic waves accelerated enzymatic fibrinolysis *in vivo* and *in vitro*, through non-thermal mechanisms including cavitation causing irreversible disaggregation of uncross-linked fibrin fibres into smaller fragments. This may be a

novel way of preventing the formation of peritoneal adhesions by performing post-operative ultrasonic sessions.

## **1.9 ADHESIONS PROPHYLAXIS**

The increased incidence of post-operative adhesions along with the substantial economic burden to society as a consequence of their complications has encouraged many researchers to look for ways of preventing or at least reducing their occurrence while retaining normal healing of the traumatised surfaces. Currently there are three main approaches of adhesion reduction or prevention: meticulous surgical technique and adjuvant therapy comprising of pharmacological agents and physical barriers.

### **1.9.1 Minimizing Surgical Trauma**

Any measure that limits surgical trauma and fibrin deposition will in theory reduce adhesion formation. Gentle tissue handling and meticulous haemostasis are necessary to prevent contact of blood with tissues. The use of talc-free gloves, non-linting cotton swabs, and finer and less reactive suture material reduces inflammatory reactions and granulomatous responses (Tol et al., 1998; Luijendijk *et al.*, 1996; Ellis, 1994). The use of reactive sutures such as chromic catgut and silk should be minimised or avoided (Ellis, 1983). Peritoneal approximation if necessary is best accomplished with small 6/0 to 8/0 monofilament sutures placed under minimal tension to minimise ischaemia. Tissue abrasion/crushing is avoided by using atraumatic micro-instruments along with the surgeon's caution in avoiding serosal injury when placing moist or dry abdominal packs. Copious irrigation during the procedure is important to avoid tissue drying (Ryan et al., 1973), to prevent blood accumulation in the peritoneum and to dilute foreign-body contamination.

### **1.9.2 Use of pharmacological agents**

The approach to pharmacological adjuvant therapy in an effort to minimise postoperative adhesions has been studied over the years using different animal models



or controlled clinical trials. However, one drawback in the development of any therapeutic agent has been the poor clinically predictive nature of animal models (Di Zerega, 1994; Wiseman, 1994). The drugs that have been tried were designed mainly to prevent the different phases of the repair pathway. The general requirements for an ideal adhesion prevention product would be that it would: (a) not interfere with wound healing; (b) not potentiate infection; (c) not evoke adhesions or fibrosis; (d) remains efficacious in the presence of blood; and (e) be degradable.

*Anti-inflammatories:* Few studies have shown a beneficial effect, with conflicting results reported after the use of intra-peritoneal steroid solution (Liao et al., 1973; Cohen et al., 1983). Furthermore, clinical use of these drugs in a postoperative situation is not without adverse reactions, such as gastro-intestinal effects (bleeding), immuno-suppression and delayed wound healing.

*Anticoagulants:* Administration of heparin intra-peritoneally in rabbits facilitated fibrinolysis and prevented postoperative adhesion formation (Diamond et al., 1991; Wiseman et al., 1993) although with some side effects such as intra-peritoneal bleeding and haematoma formation (Fukusawa et al., 1991). In a human prospective study no benefit was shown (Jansen et al., 1988).

*Fibrinolytics:* A number of fibrinolytic drugs such as plasmin, tissue plasminogen activator (t-PA), streptokinase, urokinase plasminogen activator (u-PA) have been evaluated for adhesion prevention in animal models (Montz et al., 1991; Orita et al., 1991; Wiseman et al., 1992). Tissue plasminogen activator (t-PA) and new generations of recombinant t-PA, showed some benefit in adhesion prevention, but with conflicting results of impaired wound healing, decreased tensile strength or bleeding (Menzies et al., 1992; Wiseman et al., 1994).

*Calcium Channel Blockers:* Intra-peritoneal administration of Diltiazem in rats inhibited adhesion formation to some extent, whereas intramuscular injection did not (Golan *et al.*, 1989).

*Antibiotics:* These have been used to prevent infection, thus avoiding the extensive and persistent inflammatory response and subsequent adhesion formation.

*Motility promoters:* The idea of the promoters are to prevent the contact between two traumatised opposing surfaces, by enhancing the motility of the bowel, so that the newly formed thin filmy adhesions will be broken down.

*Vitamin E:* Due to its effect on lysosomal membrane stability and anti-inflammatory properties, Vitamin E was tested for its anti-adhesiveness effect in a rat model where one group was fed orally for three weeks post-operatively and the other group was not. Although there was a trend of less fibrosis with Vitamin E, there was no statistically significant difference, and there was no difference with respect to a reduction in adhesion formation (Sanfilipo *et al.*, 1995).

### **1.9.3 Use of Physical Barriers**

Locally placed polymer-based devices that provide programmed drug delivery to a surgical site may perform better than local or systemic peri-operative doses, with systemic effects. Drugs may be incorporated into devices (films, fabrics, gels, liquids) that are used as barriers to prevent adhesions. There has been particular interest in the use of absorbable or non-absorbable membranes or agents acting as barriers applied topically in the peritoneal cavity to prevent two opposing raw surfaces from adhering to each other. The critical period for the action of these compounds is the first 5-7 days following injury. Barriers have also been used to prevent adhesion formation with more encouraging results than pharmaceuticals.

*Ringer's Lactate solution:* Had been used as peritoneal lavage at the end of surgical procedures for many years to prevent adhesion formation, but the results were not encouraging as crystalloid solutions are rapidly absorbed.

*Dextran 40 & 70% and Hyskon (Dextran 32% in 10% Dextrose):* In the peritoneal cavity, they are absorbed and metabolised slowly, thus creating mechanical separation of organs (Wiseman, 1994; Di Zerega, 1994). They act as a local anticoagulant and an osmotic agent that draws body fluids into the peritoneal cavity. This creates a floating effect, which keeps tissues from sticking together until the fluid is absorbed during the following week. Dextrans may interfere with blood group cross-matching and there is increased risk of bleeding through depletion of coagulation factors (BNF, 1999).

*Sodium Carboxymethylcellulose:* This is a highly viscous solution more slowly absorbed, less antigenic and less likely to support bacterial growth than dextran.

*Intergel (FeHA):* It is an aqueous solution composed of sodium hyaluronate ionically cross-linked with ferric chloride applied at the end of surgery. Some studies had shown its effectiveness in reducing adhesions (Wiseman et al., 1998; Thornton et al., 1998)

*Adept (4% icodextrin solution):* A new anti-adhesive product in the market that is easily applied either in open or laparoscopic procedures, but with limited clinical data to support that.

*Gore-Tex (Polytetrafluoroethylene) (PTFE):* This is a synthetic, non-reactive, non- absorbable mechanical material, which has been utilised extensively as a pericardial, diaphragmatic and peritoneal substitute. It showed conflicting results when studied *in vivo* (Goldberg et al., 1987; Boyers et al., 1988; Haney et al., 1995). The

disadvantages of this product are the presence of permanent foreign body in the peritoneal cavity and the requirement for suture fixation of the membrane (Padigas and Tulani, 1992).

*Oxidized Regenerated Cellulose (ORC) (Interceed)*: It is specifically designed to cover and separate surgically traumatised peritoneal surfaces. It becomes gelatinous within 8 hours of application and breaks down macroscopically within 3-10 days and slowly absorbed from the peritoneal cavity within 28 days of application (Wiseman, 1994). Some animal studies showed no benefit in preventing post surgical adhesion formation (Pagidas and Tulandi, 1992). Other studies and controlled multicentre trials (Wiseman, 1994) showed reduction in the extent and severity of adhesions, but clinical acceptance has been constrained by its decreased efficacy in the presence of blood or excess peritoneal fluid (Di Zerega, 1994). However when Diamond (1995) added heparin to the fabric, a significant reduction in adhesion formation was observed.

*HA-Carboxymethylcellulose (HA-CMC) (Seprafilm)*: HA-CMC is a bioresorbable membrane composed of hyaluronic acid and carboxymethylcellulose, which is non-toxic and non-immunogenic and biocompatible. It turns to a hydrophilic gel within one day of placement and protectively coats traumatised tissues up to seven days during the re-epithelialisation phase. It is cleared from the body within 28 days and is effective in the presence of blood (Burns et al., 1995; Diamond, 1996). Beck and colleagues in 1997 showed it was safe and had significantly reduced the rate of adhesion formation by nearly 50%. Other authors through clinical evidence (Singh et al., 2000) recommended the use of HA-CMC (Seprafilm) in humans, as it is superior to other available products. Wietske et al. (2002) reported that it was effective in reducing the severity of post-operative adhesions, but not the incidence, while The Cochrane collaboration (2004) showed no evidence of its effectiveness in preventing adhesion formation.

*Polylactide Resorbable Film*: A resorbable film used as a barrier to prevent post-operative adhesions in animal models with encouraging results in reducing posterior dural adhesions in the spine following laminectomy (Klopp et al., 2004).

Table 1.1 summarises the modalities of adhesion prophylaxis and prevention that have been used so far, but overall there is no treatment whether a drug or a barrier that has had a significant effect in reducing the incidence of adhesion formation in humans.

#### **1.10 Aims of this thesis**

Post-operative peritoneal adhesions occur in the majority of patients that undergo abdominal surgery and lead to complications such as abdominal pain, infertility in women and intestinal obstruction. However, the structure and pathogenesis of adhesions is still not clear and no treatment strategy shows 100% efficacy. Persistence of fibrin between damaged tissues, due to impaired plasminogen activator activity, is proposed to be the mechanism underlying adhesion formation following surgery. Yet which plasminogen activator plays a leading role and whether this same mechanism is involved in adhesion formation following an inflammatory episode is not known.

This thesis aims to initially assess the structure of peritoneal adhesions in humans and their development in an experimental murine surgical model (Chapter 2). This study may show novel features within adhesions and confirm whether they represent avascular and acellular scar tissue as has been suggested. Furthermore, by comparing human adhesions with those produced in a murine model, we will demonstrate that the experimental model is representative of the clinical situation. The second aim is to characterise nerve fibres in human adhesions and to assess their ingrowth in adhesions formed in the experimental animal model (Chapters 3 and 4). It has been suggested that adhesions may be the source of pain experienced by some patients following surgery however, a thorough assessment of the types of nerves, in

particular non-myelinated sensory nerves, within adhesions is lacking. The final aim is to further elucidate the role of fibrinolysis in adhesion formation using transgenic mice deficient in tissue plasminogen activator (t-PA) or urokinase plasminogen activator (Chapter 5). This study will allow the action of each protease to be clearly defined and may suggest better future treatment strategies.

DRUGS	<p><u>ANTICOAGULANTS:</u>  Dicumarol  Heparin  Hirudin</p> <p><u>ANTI-INFLAMMATORY:</u>  Anti-histamines (e.g. promethazine)  Corticosteroids  Nonsteroidal anti-inflammatory drugs (NSAIDS)</p> <p><u>CALCIUM CHANNEL BLOCKERS:</u>  Diltiazem  Nifedipine  Verapamil hydrochloride</p> <p><u>CYTOSTATICS:</u>  5-fluorouracil</p> <p><u>PROTEOLYTICS:</u>  Hyaluronidase  Pepsin  Plasmin  Streptokinase  t-PA  Trypsin  Urokinase</p> <p><u>MOTILITY PROMOTERS:</u>  Cisapride  Neostigmine</p>
BARRIERS	<p><u>MACROMOLECULAR SOLUTIONS AND OTHER INTRA-ABDOMINAL INSTILLATIONS</u>  Agar  Albumin  Camphor oil  Carboxymethylcellulose (CMC)  Chondroitin sulphate  Dextran (32% dextran 70 in 10% dextrose (Hyskon))  Fat  Gum Arabic  Hyaluronic acid phosphate buffered saline solution (Sepracoat)  Olive oil  Paraffin  Phospholipids (e.g. phosphatidylcholine)  Povidone iodine/polyvinylpyrrolidone (PVP)  Silicone  Vaseline</p> <p><u>MECHANICAL BARRIERS</u>  Amnion  Bladder  Cattle, fish or shark peritoneum  Cellulose  Corpus vitreum  Expanded polytetrafluoroethylene (e-PTFE) (Preclude/Gore-tex)  Gelatin  Hyaluronic acid-carboxymethylcellulose (HA-CMC) (Seprafilm)  Metal foil  Omentum  Oxidized regenerated cellulose (Interceed)  Rubber plates  Silk leaves</p>
MISCELLANEOUS INTERVENTION	<p><u>ULTRASOUND VIBRATIONS</u> (to stimulate motility)</p> <p><u>IRON SHAVINGS</u> (+magnesium)</p>

**Table 1.1** Modalities in Adhesion prophylaxis and prevention (Modified from Risberg, 1997)

## **CHAPTER TWO**

### **A LIGHT MICROSCOPIC AND ULTRASTRUCTURAL ASSESSMENT OF PERITONEAL ADHESIONS IN A MURINE MODEL**



## **2.1 INTRODUCTION**

The peritoneum is lined by the mesothelium, which allows the viscera to slide freely against each other and the abdominal wall. Loss of mesothelial integrity after surgery or inflammation may induce a sequence of events which leads to fibrous adhesion formation between damaged surfaces and this is prevented when the mesothelial cell lining is maintained (Haney, 2000). The complexity of adhesion formation and limited understanding of its pathogenesis has hampered the development of satisfactory treatment.

Indeed only a few histological and ultrastructural studies have been conducted in different species and experimental model systems as well as limited time points making them difficult to compare (Ellis, 1962; Schade and Williamson, 1968; Watters and Buck, 1973; Milligan and Raftery, 1974; Bakkum et al., 1995). In general these studies have shown that following trauma peritoneal healing begins within minutes of injury. The combination of coagulation and suppression of fibrinolysis leads to the formation of a provisional matrix or fibrin clot between damaged surfaces. The fibrin clot is a complex macro-molecular substrate for cell migration and contains a wide spectrum of factors and chemo-attractants for the ingrowth of variety of cell types. In the presence of inflammatory cells, predominantly neutrophils and macrophages over the initial three days the fibrin clot is gradually replaced by granulation tissue, with the formation of new blood vessels and with invasion of motile fibroblastic spindle-shaped cells. At this stage deposition of extracellular matrix (ECM) results in a fibrinous structure being converted into a fibrous adhesion usually within 5-7 days. Mesothelial cells cover the adhesion forming a continuous basement membrane which becomes permanent within 7 days (Eskeland, 1966; Hubbard et al., 1967; Raftery, 1973).

The aim of this study was to assess the development of an adhesion, using a range of techniques including light, scanning (SEM) and transmission electron

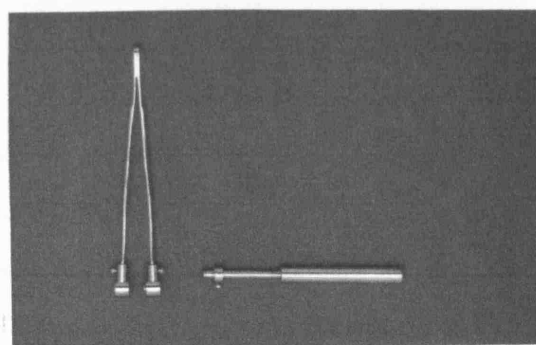
microscopy (TEM). Adhesions were induced using a standardised surgical trauma in a murine model and formation assessed over a time course of six hours to six months post-surgery. Most previous studies have assessed adhesions over a very short period of time (1-2 weeks) and by using a single technique. Furthermore, often the experimental procedure used has not been reproducible or site specific. Using a variety of approaches and a standardised model six months post surgery, will provide greater detail at the cellular level of the pathogenesis of adhesion formation.

## 2.2 MATERIALS AND METHODS

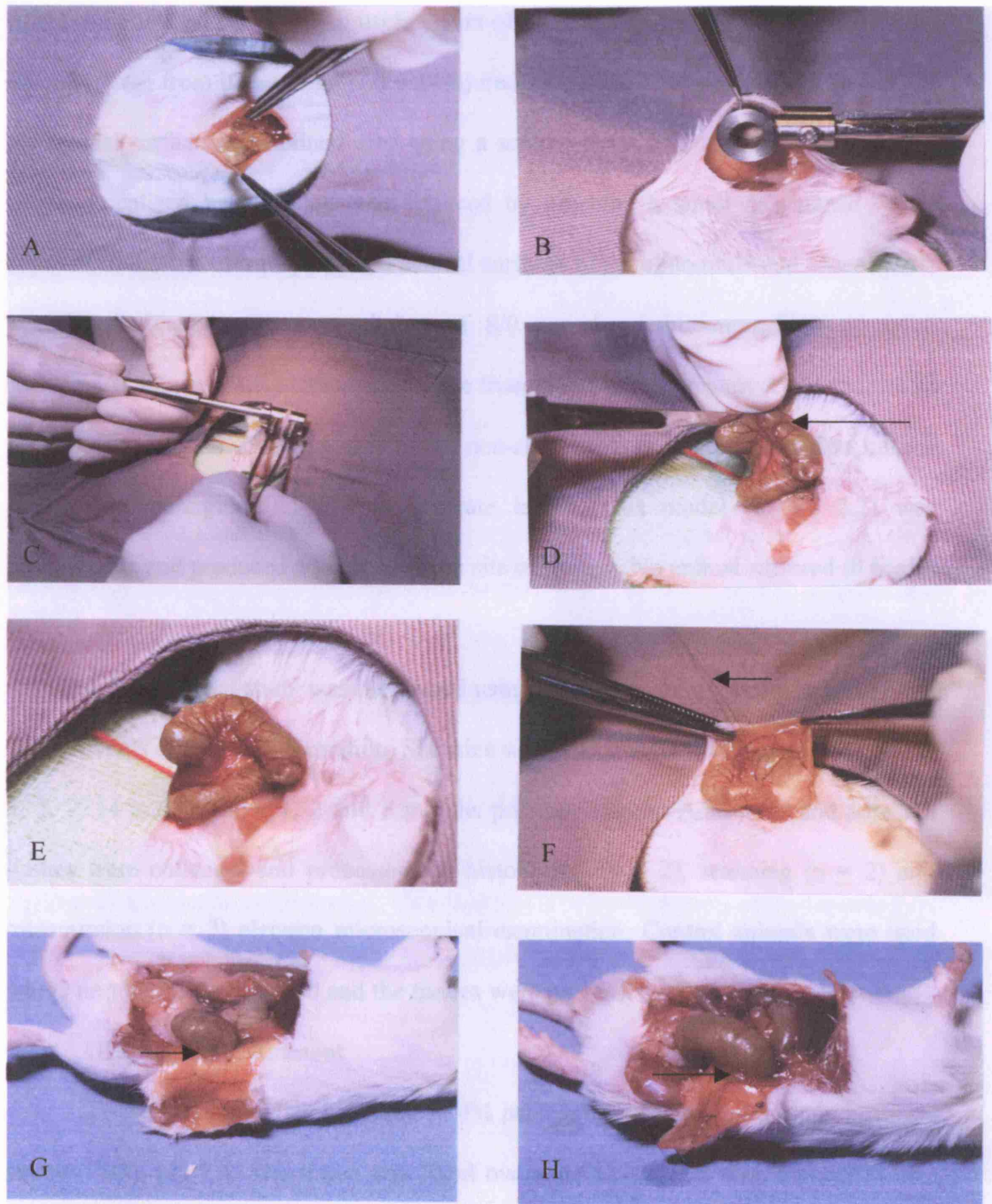
All chemicals were obtained from Sigma (Poole, Dorset, UK) or BDH (Lutterworth, UK) unless otherwise stated.

### 2.2.1 Murine post-operative peritoneal adhesion model

A murine post-operative adhesion model was developed as part of my M.Sc. Project (Sulaiman, 1997). The model produces reproducible site-specific post-operative peritoneal adhesions. All studies described were performed using this model some with slight modifications (mentioned later). Home Office license requirements were fulfilled for the project and personal use. Sixty-six male Balb-c mice, 8-12 weeks old with average weight of 30 grams were used for the study (Harlan Ltd, Bicester, UK) were operations under general anaesthesia induced with 4% halothane delivered in a mixture of nitrous oxide (1.5L/min) and oxygen (3L/min) under aseptic conditions. The mice were shaved and the abdomen was opened by a midline incision. A standard site on the left lateral abdominal wall midway along and 1cm lateral to the midline incision was clamped and then injured by three rotations of the rod of a trauma instrument (Figures 2.1 and 2.2) manufactured by M. Eastwood, University of Westminster, UK. This instrument delivered a precise size and depth of injury to the mesothelial,



**Figure 2.1:** Trauma instrument



**Figure 2.2:** The surgical procedure: (A) mid-line incision, (B) & (C) application of the trauma instrument and injury to the abdominal wall. (D) scraping the caecum, (arrow), (E) haemorrhage from a mesenteric blood vessel. (F) approximating the surfaces with stitches (arrow), (G) & (H): one-week post surgery, adhesions have developed (arrow). (Reproduced from MSc thesis in Surgical Science; Sulaiman, 1997)

submesothelial and underlying muscle layers of the abdominal wall. The surface of the caecum, 1 cm from its terminal end was injured by standardized scraping (30 times) of the serosal surface at a defined area using a scalpel blade (no. 15) and irrigated with saline. Localized haemorrhage was induced by pinching a small mesenteric vessel adjacent to the site of injury. Injured serosal surfaces of the abdominal and caecal walls were approximated with two stitches of 8/0 non-absorbable monofilament nylon (Ethicon, Edinburgh, UK) either side of the trauma site approximately 1 cm apart. The peritoneum and skin were closed with 6/0 non-absorbable monofilament nylon sutures (Ethicon, Edinburgh, UK) as two separate layers. This model (Figure 2.2) was reproducible and produced adhesions at the site of trauma. No animal suffered ill health or died.

A longitudinal study was performed using this model to assess the temporal and spatial events of adhesion formation. Six mice were killed at each time point: 6hrs, 1, 2, 3, 5, 7, 14 and 21 days, 1, 2 and 6 months post-operatively. Adhesions and adjacent tissues were collected and processed for histological (n = 2), scanning (n = 2) and transmission (n = 2) electron microscopical examination. Control animals were used where no trauma was induced and the tissues were processed as below.

### **2.2.2 Histological assessment**

Tissue was immediately placed in 4% paraformaldehyde in phosphate buffered saline (PBS), pH 7.4. The tissue was fixed overnight at 4°C and then embedded into paraffin wax in an automated tissue processor (Shandon Citadel 2000, UK) orientated in a wax block using a Tissue Embedding System (Shandon Tissue Tek Histocentre 2, UK). Tissue was sectioned at 5 µm using a microtome (Pabisch, Germany) and serial sections collected. Two different staining methods were used; Haematoxylin & Eosin and Masson's Trichrome (see Appendix 1). Histological examination of the centre of adhesion (4 sections per block) was performed using an Axiophot light microscope

(Zeiss, Germany). Photographs were taken with Kodak Ektachrome 64 tungsten colour reversal film.

### **2.2.3 Transmission Electron Microscopy**

Adhesion specimens were fixed in a mixture of 1% paraformaldehyde and 5% glutaraldehyde in 100 mM sodium cacodylate buffer, pH 7.4 overnight at room temperature. They were then washed in fresh 100 mM sodium cacodylate buffer and fixed in 1% osmium tetroxide in 100mM sodium cacodylate buffer, pH 7.4 for 1hr. After re-washing in cacodylate buffer, specimens were further stained in an aqueous saturated solution of uranyl acetate for 1hr, followed by dehydration through a series of alcohol concentrations (50%, 70%, 90% & 100%), and embedded in an epoxy resin (Araldite). For light microscopy, semi-thin sections 0.5-1µm were cut with a microtome (Reichert, Austria). Sections were floated on a drop of distilled water and dried onto a glass slide heated to 50°C. Sections were stained with 1% alcoholic toluidine blue and mounted with a drop of Araldite resin. Sections were examined under a Zeiss Axiophot light microscope (Zeiss, Germany) equipped with phase contrast optics and photographed using Kodak Ektachrome 64 ASA tungsten colour reversal film. For transmission electron microscopy, ultra-thin sections (50~100nm) were collected on copper grids and stained with 3% uranyl acetate in 50% ethanol with lead citrate. The sections were viewed with a Philips 400 transmission electron microscope (Eindhoven, Holland) and photographed with Kodak electron microscope film 4489.

### **2.2.4 Scanning Electron Microscopy**

Adhesion specimens were fixed with a mixture of 1% paraformaldehyde and 5% glutaraldehyde in 100 mM sodium cacodylate buffer, pH 7.4 overnight at room temperature. They were then washed in fresh 100Mm sodium cacodylate buffer (3 changes/5 minutes each) followed by 1% aqueous solution of osmium tetroxide (1 part osmium tetroxide plus 3 parts of sodium cacodylate buffer pH 7.4) for 30 – 90 min. in the dark, then re-washed with fresh 100 Mm sodium cacodylate buffer (3 changes/5

minutes each). Samples were fixed with 2% tannic acid for 1 hr, washed with fresh cacodylate buffer and dehydrated with a series of alcohol concentrations: 50%, 70%, 90% (10 min each) and lastly absolute alcohol for 20 min. Specimens were then immersed in neat amyl acetate for 10 min.

Critical point drying was performed using a CPD 020 apparatus (Balzers Union, Lichtenstein) according to manufacturer instructions. The chamber containing the coated samples was filled with CO<sub>2</sub> to replace the dehydrating solvent and then emptied for up to 3 cycles. The temperature was set at 40°C and the pressure kept at 80 bars. Drying occurred when critical point for CO<sub>2</sub> (72.9 bar) and 31.1°C was reached.

Samples were then placed on aluminium stubs in a vacuum chamber in the gold coating apparatus (Polaron Equipment Ltd., SEM coating unit E 5000, UK). After switching on the rotary pump and sputter coater, Argon gas was flushed through the chamber. As the vacuum reached <0.1 Torr, Argon gas leak valve was adjusted until the monitor reading showed 600KV and 20 milliAmps, then the timer was activated (4.5 minutes/cycle). For an average coating, 2 cycles were needed. The specimens were examined under a scanning electron microscope (S-530 SEM, Itachi, Japan) and photographed with ILFORD PF4 120 film.

## **2.3 RESULTS**

All animals survived and remained healthy throughout the study and no death was recorded. Macroscopic and histological assessment was previously described (Sulaiman, MSc. Thesis, 1997), however, key results and images are summarized for clarity.

### **2.3.1 Macroscopic Assessment**

Post-operative peritoneal adhesions consistently formed at the site of injury between the caecum and the anterior abdominal wall. In 66 operated mice, 24 also showed a pelvic fat body adherent to the midline incision, 14 to the site of trauma and 8 to both the trauma site and midline, 6 hours post-surgery. Coagulation had occurred and a fibrin clot was detected between injured surfaces at 6 hours. This structure was friable causing difficulty in tissue collection. By day 2-5 gel-like fibrinous adhesions had formed where fibrin threads extended between opposing peritoneal surfaces at the trauma site. Minimal traction resulted in detachment from between serosal surfaces. At 7 days fibrous adhesions were evident between the caecum and abdominal wall and needed more traction to separate. There was continuity in serosal integrity between the adhesion and adjoining structures by this stage (Figure 2.2G and H). From 14 days to 6 months peritoneal adhesions retained the same appearance. Few animals also displayed fibrous adhesions in areas distant to the site of trauma such as fat body to the spleen and liver and omentum to small bowel and pelvic wall. One animal was excluded from the study as it developed a matted mass enclosing the caecum, seminal vesicles, small bowel and abdominal wall. Adhesions (n=6) were collected at set times and assessed histologically and ultrastructurally

### **2.3.2 Histological Assessment**

*Six hours-24 hours after injury*, both trauma sites were covered by a fibrinous exudate with acute inflammatory cells (Figure 2.3A). The mesothelial, submesothelial

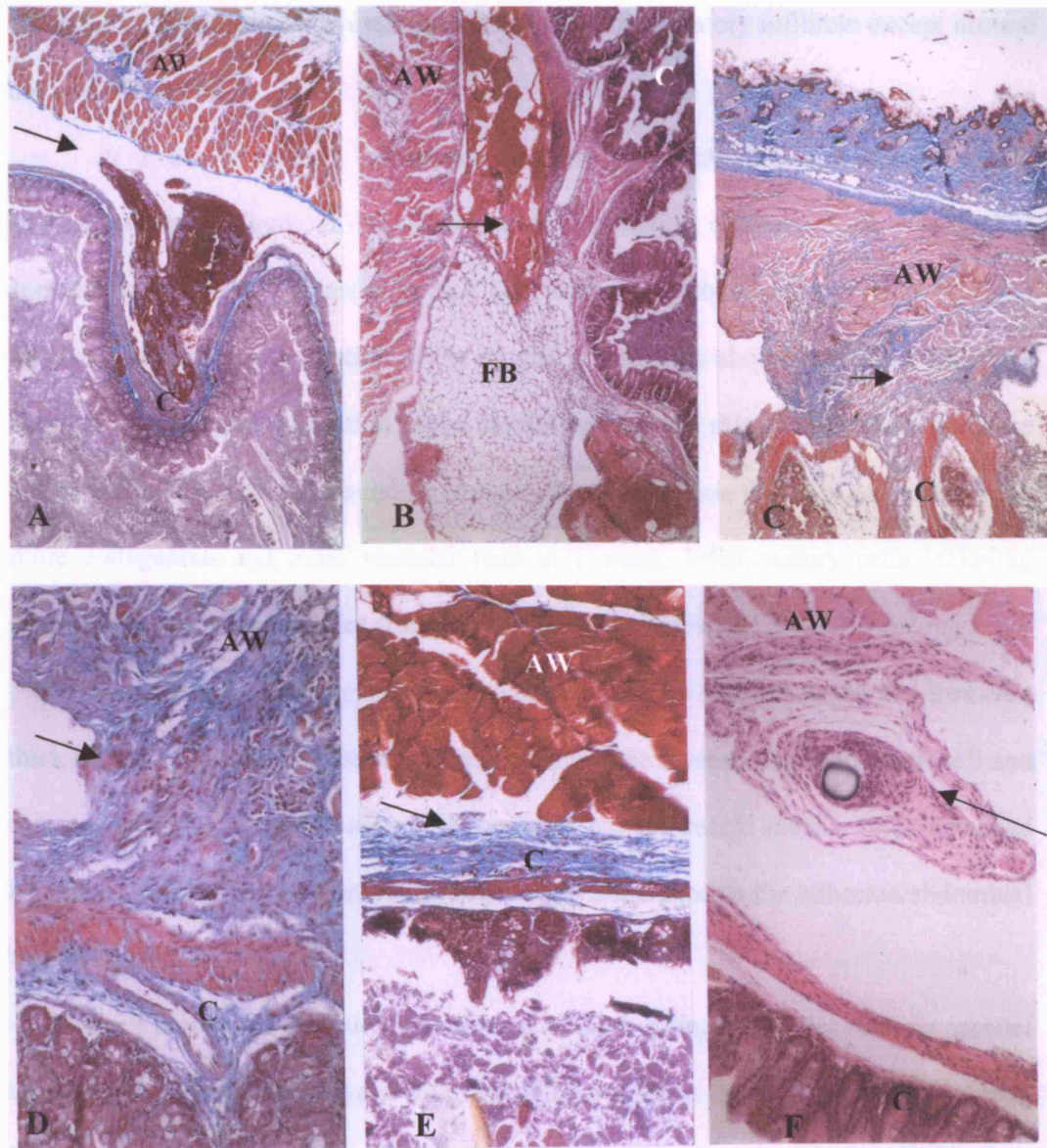


and the skeletal muscle layers were disrupted. Within the fibrin mesh there were many cell types, including inflammatory cells some cells that were not clearly identifiable. The pelvic fat body was adherent to both the denuded serosal surface and the fibrinous exudate as early as 6 hours following surgery (Figure 2.3A and B) in some animals.

*Day 2*, there were inflammatory cells present at trauma site as well as uncharacterised cells. The outer muscle layer of the abdominal wall showed necrosis and haemorrhage and the submucosal layer of the caecum displayed dilated vessels and focal haemorrhage. An extensive acute inflammatory response was associated with the disrupted submesothelial and muscle layers especially around the stitches.

*Three-four days after injury*, the damaged serosal surfaces were tethered together by granulation tissue composed of inflammatory cells and strands of collagen within the fibrinous exudate. Fibroblast-like cells were present combined with collagen and small blood vessels dispersed within the adhesion matrix. The disrupted muscle of the abdominal wall was involved in the developing adhesion. Inflammatory cells were reduced and only cells of the initial inflammatory reaction that remained elevated were round cells thought to be macrophages or monocytes. When the pelvic fat body was involved in the developing adhesion a moderate inflammatory infiltrate (mainly neutrophils and macrophages) infiltrated deep into the adipose tissue as well as along the injured serosal surfaces around the suture lines.

*Five-seven days post-surgery*, adhesions were well formed (Figure 2.3C), highly cellular and vascularised. Typical of granulation tissue, they were composed of fibroblasts embedded within randomly arranged collagen fibres. Large blood vessels were found where the fat body had joined the adhesion site. Muscle fibres in the body wall were intertwined with collagen fibres adjacent to the adhesion site. Mesothelial integrity was established at 5 days, the surface of the fibrous adhesion was lined with a flattened layer of cells that was continuous with the surface of the caecum and parietal



**Figure 2.3:** Histological assessment of post-operative adhesions. (A) Six hours post-surgery, a fibrin clot (arrow) was present between the abdominal wall (AW) and caecum (C) (x450); (B) One day post-operatively, fat body (FB) was adherent to remnants of fibrin clot (arrow) (x600); (C) One week post-surgery, the adhesion was fully developed (arrow), and continuous with abdominal wall and caecum (x320); (D) Two weeks post-surgery, cellular adhesion (arrow) (x600); (E) Four-weeks post-surgery, showing reduced cellularity (x480). (F) Inflammatory cells around a stitch (arrow) one month post-operatively. (x420). A, C, D & E (Masson's trichrome), B & F (Haematoxylin & Eosin).

peritoneum. There was an overall decrease in the inflammatory infiltrate except around the suture material.

*At 2 weeks*, mature adhesions were found attaching the caecum to the abdominal wall (Figure 2.3D). There were no major differences in histological appearance from day 7. Mast cells and fibroblasts were abundant within the collagen bundles. There was regeneration of the muscle layer of the abdominal wall with fibres arranged in a parallel orientation to the dermal layer of the skin. The granulation tissue within the adhesion was replaced by mature connective tissue that was less cellular but more collagenous and more vascular than at 1 week. Inflammatory cells including macrophages were sparse apart from around the suture lines.

*Three weeks following surgery*, the adhesion tissue had contracted and formed a thick band of collagenous material between the muscle layer of the abdominal wall and caecum. In some samples during tissue processing, the caecal muscle layer detached from the submucosal layer probably due to tight adherence to the adhesion/abdominal wall component.

*By 1 month*, the adhesion was formed of thick collagen bundles running parallel to the muscle and dermal layers with low cellular content that was mainly fibroblasts (Figure 2.3 E). Neuronal elements were observed within the adhesion usually in close proximity to the blood vessels. Acute inflammatory cell response was less evident but giant cells and macrophages were present around the suture material in association with fibroblasts and collagen that were arranged in a concentric pattern (Figure 2.3F).

*From 3 to 6 months*, the histological findings of the adhesion were similar to those seen at 1 month with few inflammatory cells in a mature contracted collagenous matrix.

### 2.3.3 Transmission electron microscopy

Transmission electron microscopy defines cellular details at high magnification and high resolution but with limited area. It was desirable to assess the whole adhesion tissue joining both serosal surfaces, as well to focus in to elucidate cell types. Therefore, semi-thin sections were initially prepared for light microscopy where the adhesion could be studied as a whole. Subsequently, ultra-thin sections were collected for electron microscopy from the same block after trimming them down around the selected area of maximum interest. Thus micrographs were taken of the middle of an adhesion and in some specimens the edge of the adhesion was also examined.

*Six hours-24 hours:* there was loss of the mesothelial cell surface and disruption of the sub-mesothelial and muscular layers at the trauma site (Figure 2.4A). Fibrin strands, collagen and elastin fibres were abundant between the damaged serosal surfaces (Figure 2.4B). A variety of inflammatory cells were embedded in the fibrin matrix (Figure 2.4C and Figure 2.5) predominantly PMNL showing phagocytosis of necrotic cells (Figure 2.4C), macrophages, mast cells and eosinophils, as well as apparent cell-cell contact between inflammatory cells (Figure 2.5).

*Two days after injury,* fibrin remained extensive at the trauma site. Macrophages exceeded PMNLs and a scatter of eosinophils was present throughout the damaged peritoneum. There were fibroblast-like cells and collagen fibrils as well as a variety of cells of unknown identity. There were no mesothelial cells on the surface of the developing adhesion. In areas of the adhesion there were fibroblasts and bundles of collagen (Figure 2.6) and in other area there was still fibrin remained as well as macrophages and a few lymphocytes and mast cells.

*By 3-4 days,* most of the fibrin had disappeared and larger numbers of cells that resembled fibroblasts with collagen fibrils at the site of trauma. They were elongated, flat and contained an oval nucleus with a thin regular rim of chromatin and prominent

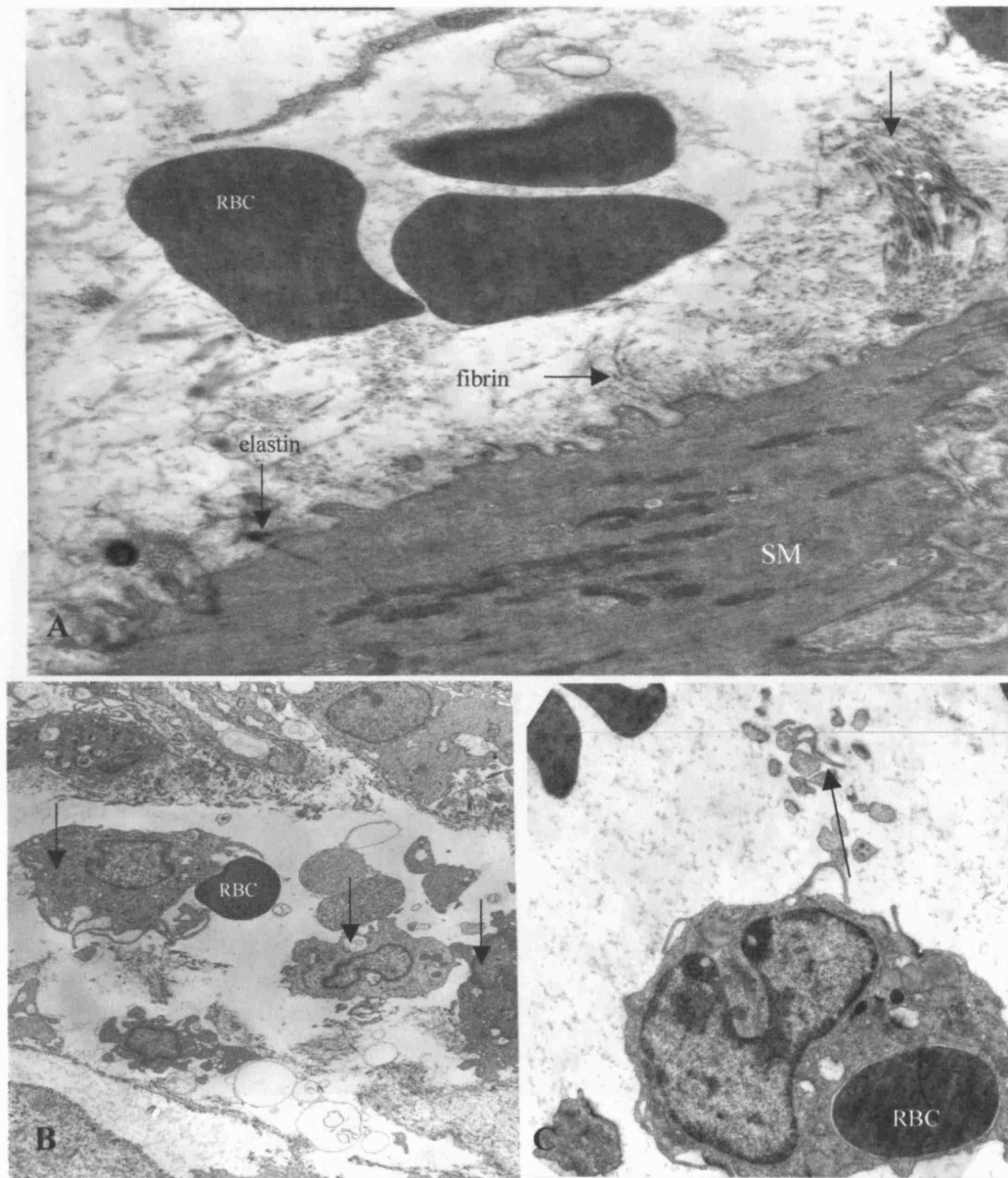
nucleolus. The endoplasmic reticulum network was evident, as well as the Golgi apparatus and mitochondria. Cells found on the adhesion surface were identified as cells of the monocyte/macrophage lineage. Occasional capillaries were apparent.

*Through days 5-7*, a greater number of fibroblasts were found with further collagen deposition and organization (Figure 2.7 and Figure 2.8). Other cells included mesothelial cells (Figure 2.9) macrophages and eosinophils, blood vessels with endothelial cells showing elongated nuclei with prominent thin regular rim of chromatin containing erythrocytes were noted in the middle of adhesion. Some blood vessels were surrounded by inflammatory cells and by semi-lunar elongated cells that may have been pericytes (Figure 2.10A). There was no evidence of media or adventitial layer formation suggesting early development. By day 5 post-surgery, structures were detected that resembled neuronal tissue with axon-like fibres (Figure 2.12A) and they appeared nearer to the abdominal wall than the caecal wall at the adhesion site.

*At 2-3 weeks*, the relatively few cells present were predominantly fibroblasts with a few macrophages and mast cells remaining. Collagen fibrils were abundant and blood vessels (Figures 2.8 and 2.10 B) well developed and the cytoplasmic membrane of the endothelial cell was lined with numerous villi. At this stage, neuronal tissue was more developed assuming the shape of a nerve bundle, with axons and surrounding Schwann's cells and myelin sheath. There was dense collagenous tissue deposition around the neuronal tissue (Figure 2.12B).

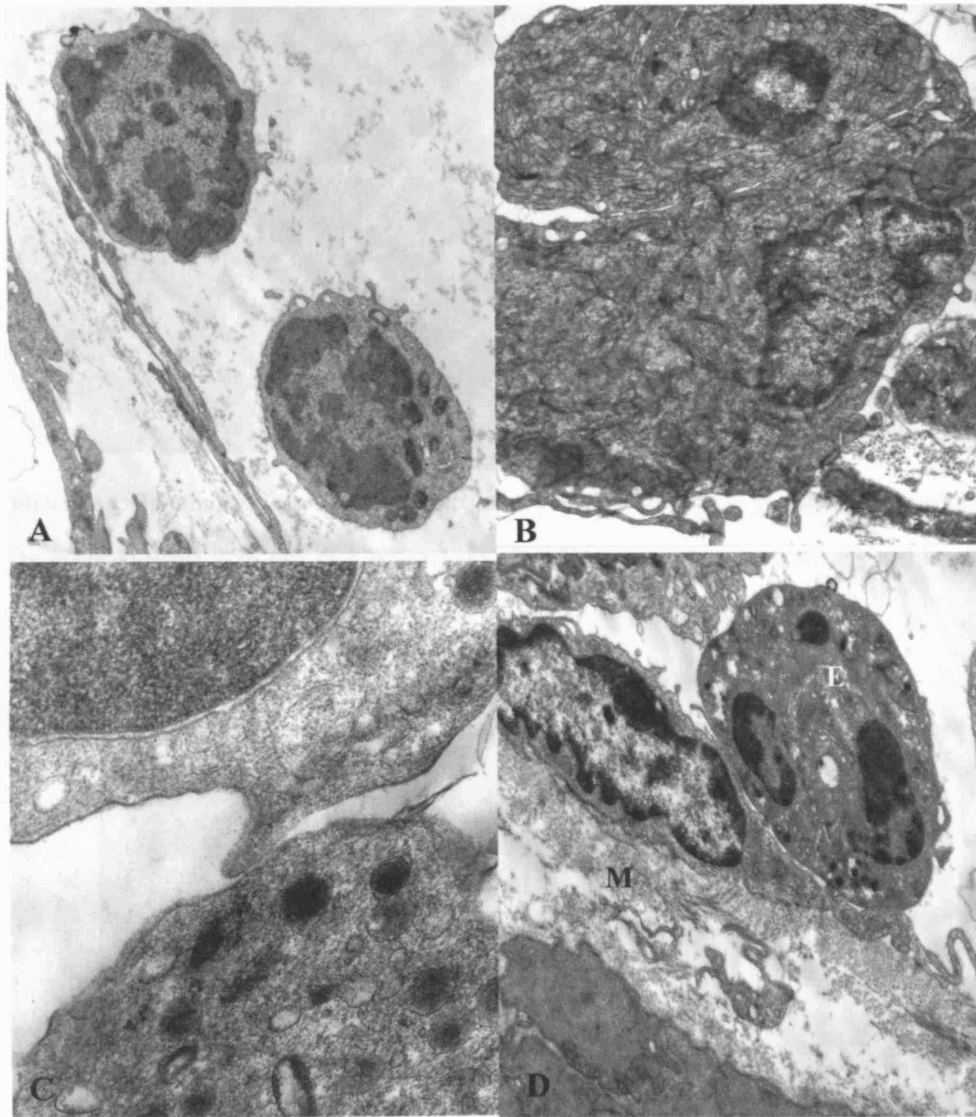
*One month-six months after injury*, at one month adhesions showed the same appearance as 2 weeks post injury, with blood vessels surrounded by spindle-shaped fibroblasts, mast cells and bundles of collagen (Figure 2.10). A nerve bundle was detected in semi-thin sections in the middle of the adhesion almost equidistant from both the caecum and the disrupted muscular layer of the abdominal wall (Figure 2.11). Transmission electron microscopy of the ultra-thin section of the nerve bundle

demonstrated a perineurium surrounding multiple myelinated nerve fibres and un-myelinated fibres with accompanying Schwann cells. The un-myelinated axons appeared to have a smaller diameter than the myelinated axons. Serial sectioning demonstrated multiple blood vessels close to the nerve bundle, which appeared to be originating from a somatic nerve in the abdominal wall. By eight weeks, a similar finding was seen, but on this occasion, it was closer to the abdominal wall (Figure 2.12 C). Higher magnification showed the highly organized myelin sheath wrapped around the axon by the Schwann's cell, closely associated with collagen and elastic fibres (Fig. 2.12 D).



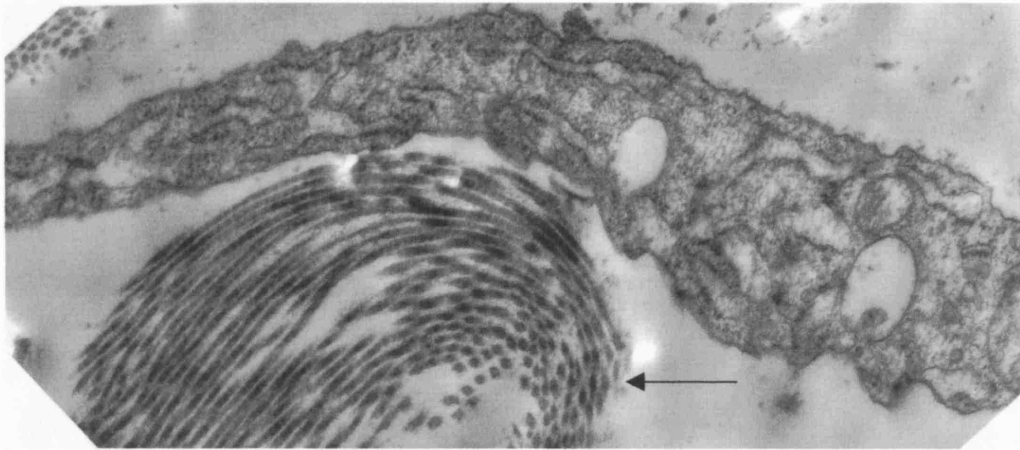
**Figure 2.4:** Transmission electron micrograph of the trauma site 6 hours post-surgery. (A) Loss of mesothelial and submesothelial layers, exposed the underlying smooth muscle (SM) with villiform-like surface. Fibrin was found at the trauma site with elastin fibres and collagen fibrils surrounding erythrocytes (RBC) (x 6000). (B): Multiple inflammatory cells (arrows) at trauma site with adjacent erythrocytes (x 8000). (C) A neutrophil with phagocytosed erythrocyte and multiple adjacent platelets (arrow) present at the trauma site (x 4800).



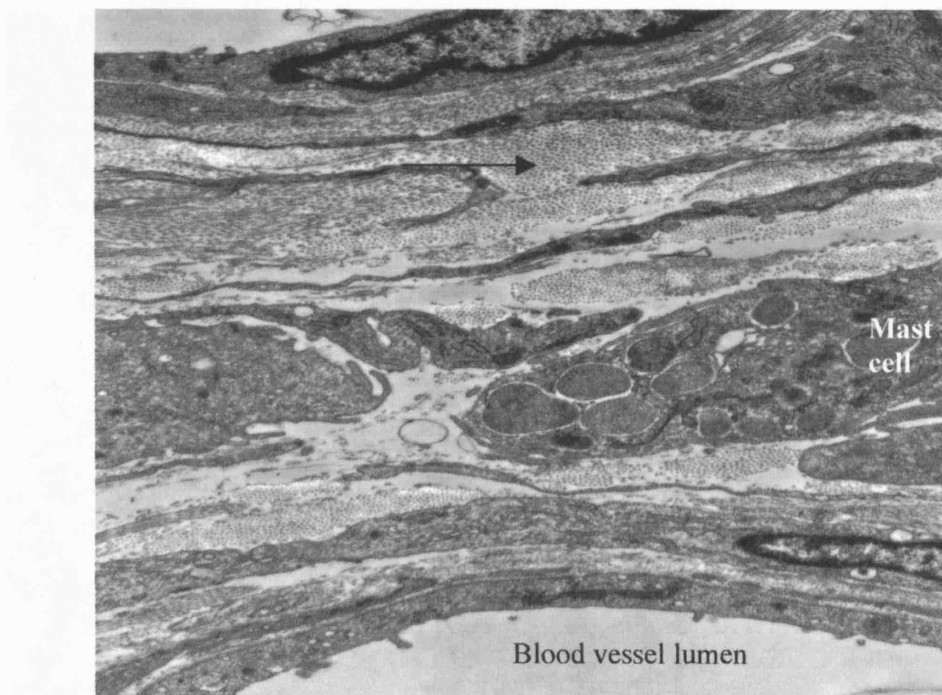


**Figure 2.5:** Micrograph of inflammatory cells at the trauma site 24 hours post-surgery. (A): Two lymphocytes (x 4000). (B): Two plasma cells with extensive development of granular endoplasmic reticulum (x 8000). (C): Cell-cell contact between two mast cells (x 40000). (D): Cell-cell contact between a macrophage (M) and eosinophil (E) (x 8000).

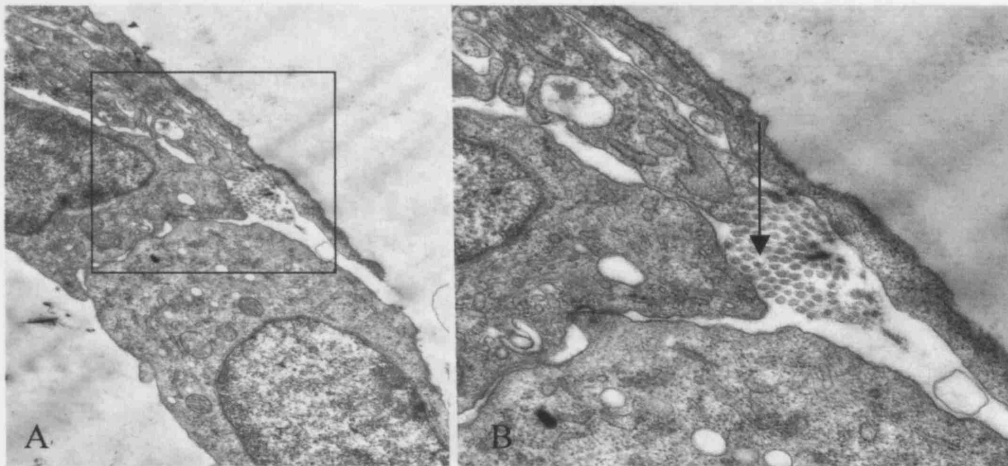




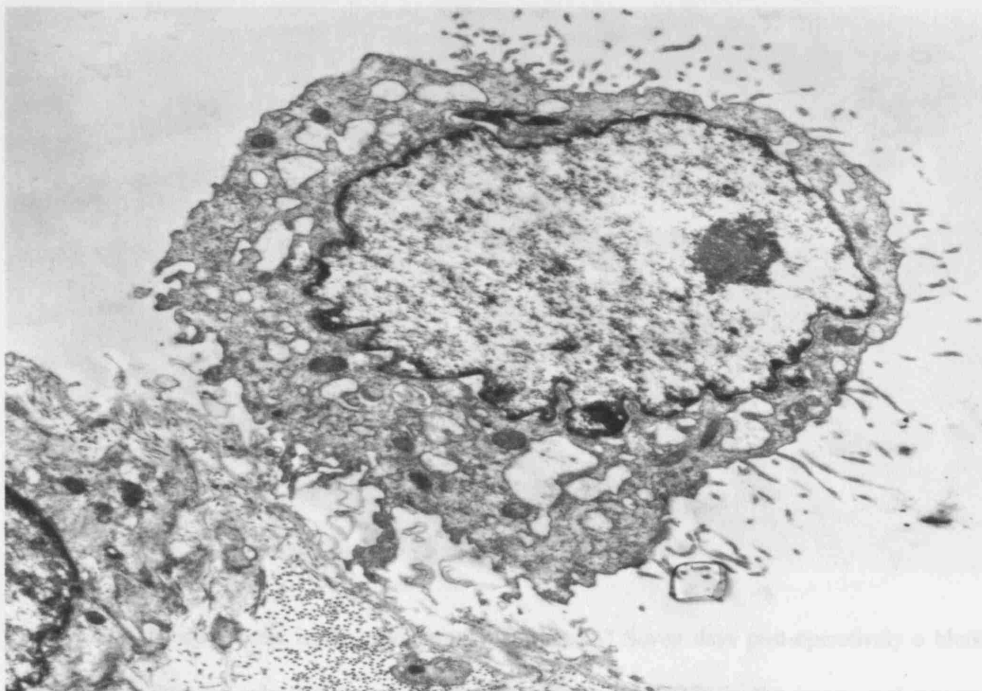
**Figure 2.6:** Transmission electron micrograph of a fibroblast adjacent to collagen fibrils (arrow) within the adhesion tissue from two days post-surgery. The fibrils are cut cross-sectionally and longitudinally with cross-banding (x22000).



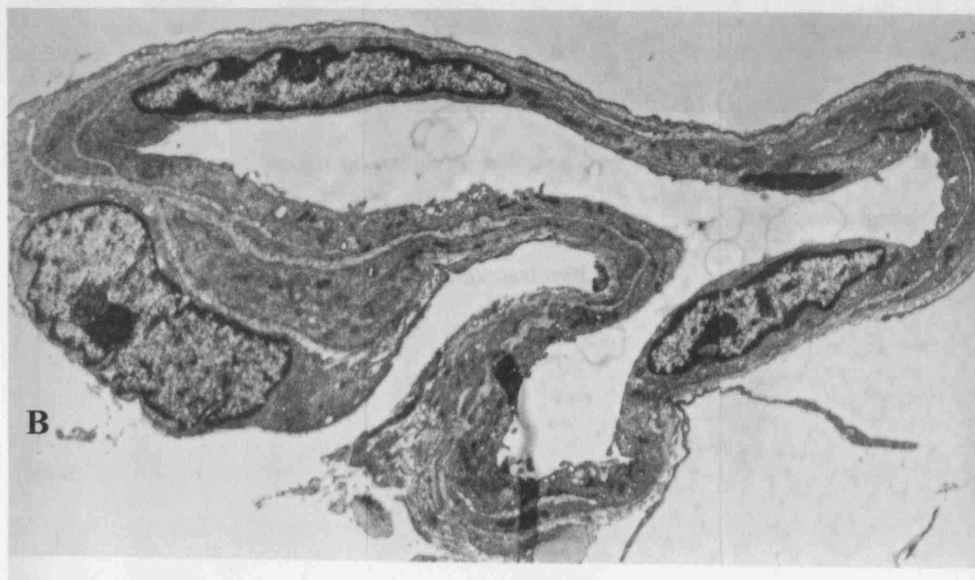
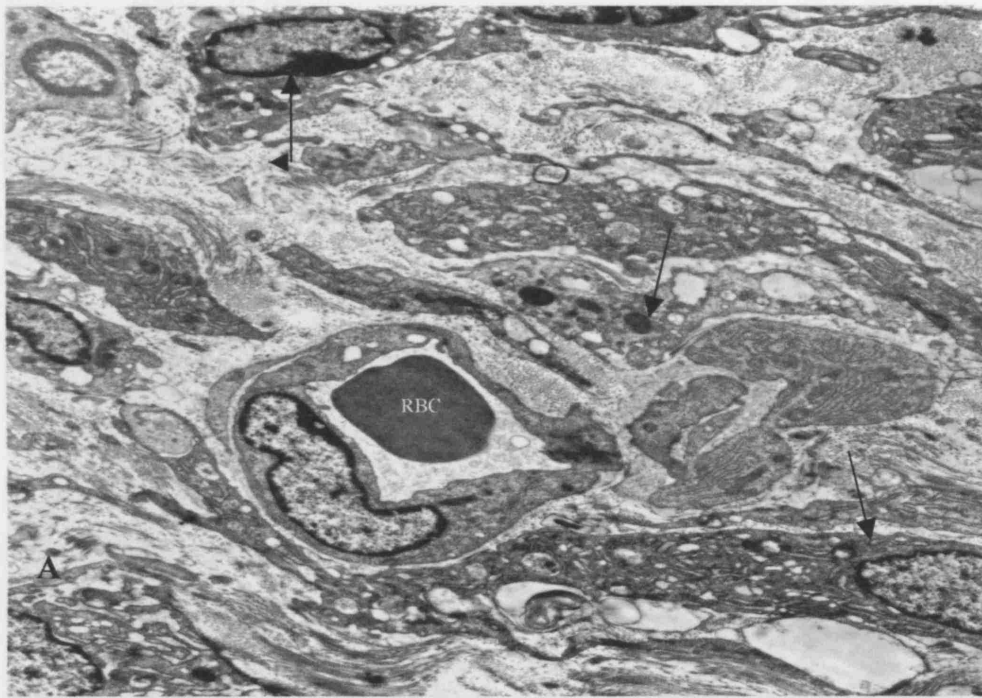
**Figure 2.7:** Transmission electron micrograph of adhesion from one week post-operatively. In the middle of adhesion tissue, mast cells, fibroblasts (short arrow), collagen fibres (arrow) and blood vessels were found (x 6000).



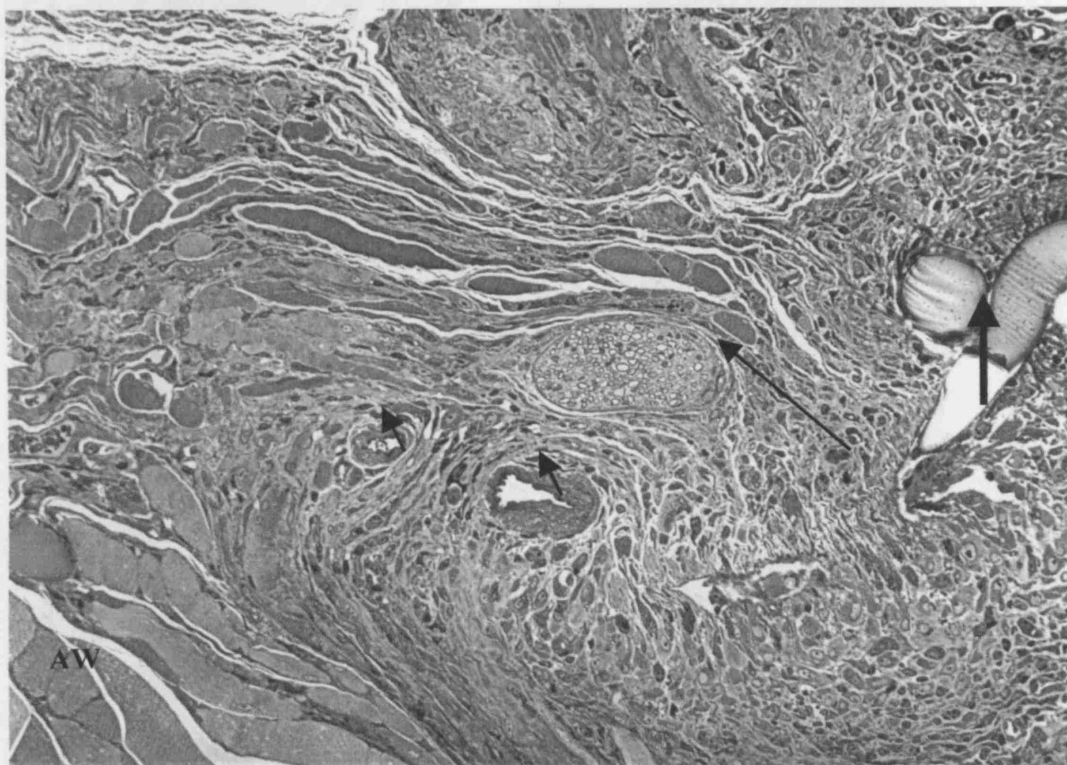
**Figure 2.8:** Fibroblast with associated collagen fibres in developing adhesion from 5 days post-surgery. (A) Two adjacent fibroblasts (x 10000). (B) Higher magnification of inset showing deposition of collagen fibres (arrow) observed next to the surface of one cell that appeared to be continuous with its cell membrane (x 20000).



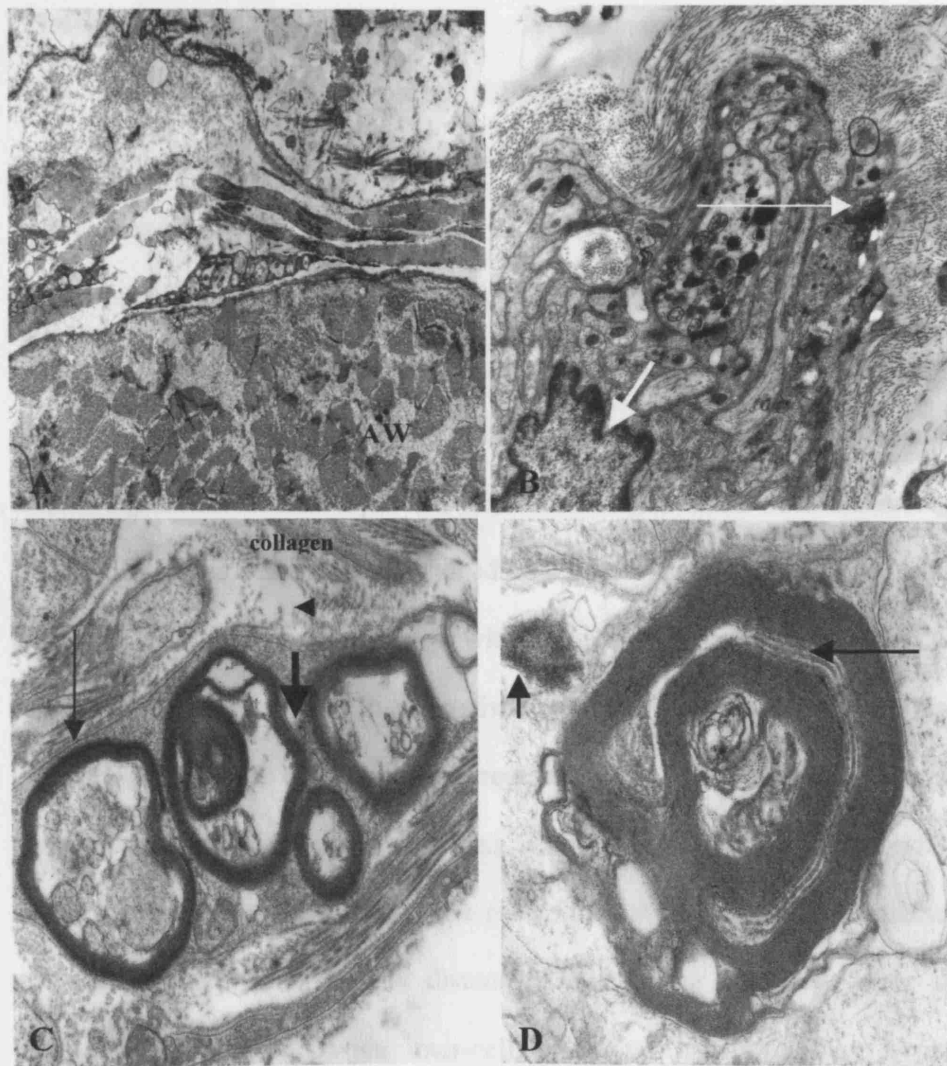
**Figure 2.9:** Surface of the adhesion at one week post-surgery, showing an adjacent mesothelial cell with abundant microvilli (arrow) covering its surface (x 4800).



**Figure 2.10:** Blood vessels in the developing adhesion (A) Seven days post-operatively a blood vessel was found within the adhesion tissue displaying erythrocytes (RBC) in the lumen and surrounded by inflammatory cells (arrows) and collagen fibrils (x 3700); (B) Two weeks post-operatively; a blood vessel was found within the adhesion showing endothelial cells resting on a basement membrane (x 6000).



**Figure 2.11:** Semi-thin section of peritoneal adhesion one month post-operatively, in the middle of adhesion, a nerve bundle was observed (long arrow) near several blood vessels (short arrows) and a stitch (thick arrow) (x 250), Toluidine blue. AW: abdominal wall.



**Figure 2.12:** Neuronal tissue present in a developing adhesion. (A) Five days post-operatively, neuronal tissue (arrow) was found in the adhesion amid collagen fibres and inflammatory cells (x 4000). (B) Three weeks post-surgery, neuronal tissue was at an advanced stage of maturation surrounded by abundant collagen fibres. Myelin containing tissue (thin arrow) and a Schwann cell appeared in the mature shape of a nerve bundle (x 10000). (C) Eight weeks post-surgery, a nerve bundle was seen with myelinated fibres (thin arrow) and Schwann's cells (thick arrow) surrounded by collagen fibrils (x 20000). (D) Myelinated nerve fibre (long arrow) and associated elastin fibre (short arrow) (x30000).

#### **2.3.4 Scanning electron microscopy**

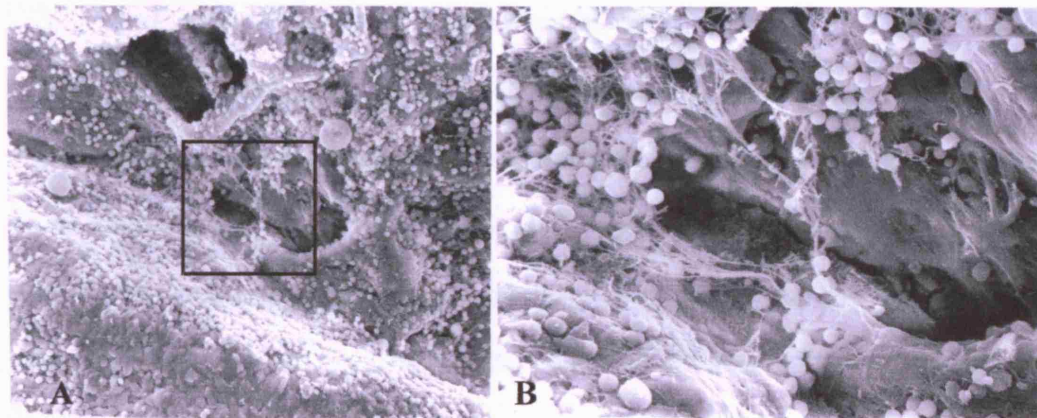
Scanning electron microscopy (SEM) permitted a more detailed analysis of the surface and the topographical arrangement of the peritoneal adhesion tissue as a whole that supported the findings previously observed with light microscopy and produced some interesting results that could not have been observed otherwise.

*Six hours-24 hours post-surgery*, there was disruption of tissue architecture with loss of the cell surface lining of the caecum and abdominal wall displaying the underlying submesothelial layer and fibrinous exudate at the trauma site (Figures 2.13 & 2.14). Tortuous fibrin threads bridged both serosal surfaces with acute inflammatory cells and erythrocytes incorporated within the fibrin meshwork (Figure 2.13A) and surrounding suture material. In comparison, with control animals no fibrin threads were detected. Many cells displaying an irregular border were found where many of the residual mesothelial cells were damaged. Several long slender filopodia extended from one cell surface to another and to the substratum, which are features characteristic of macrophages. Mesothelial cells distant to the trauma site were more prominent, cuboidal in shape with wide inter-cellular spaces indicating they were activated mesothelial cell (Figure 2.15).

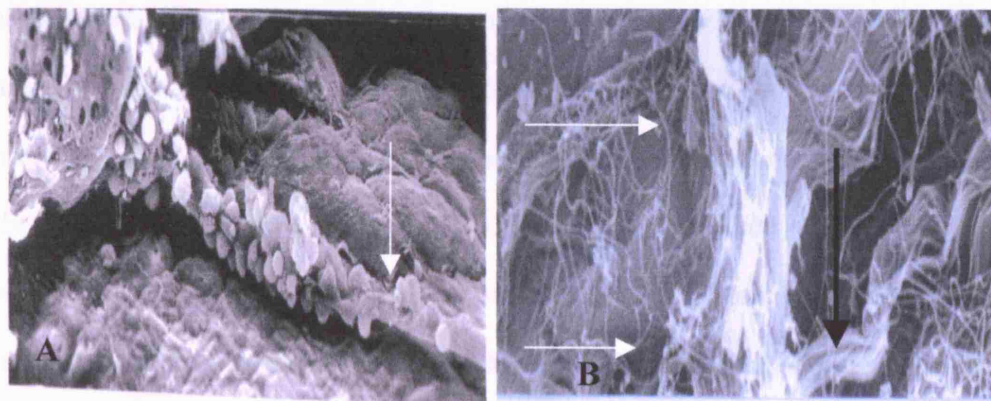
*Two days*, at the trauma site there was an inflammatory cellular response with an increased number of macrophages but other cells such as fibroblasts, mast cells and few mesothelial cells were also present. Fibrin was present at the trauma site joining both injured surfaces as in day one. Some mesothelial cells were found loosely attached to the surface of the caecal or abdominal wall displaying a single or double filipodia. It was not clear whether they were migrating or floating cells adhering to the surface (Figure 2.15). At the site of trauma and adjacent areas, multiple circular defects on the surface surrounded by various cell types were observed. At higher magnification, these



surrounding cells displayed abundant fine microvilli. Few circular areas on the undamaged body wall peritoneal lining were devoid of inflammatory cells, and the



**Figure 2.13:** Scanning electron micrograph of the trauma site six hours post-surgery. (A): Inflammatory cellular infiltrate embedded in fibrin threads (x 600). (B): Higher magnification of the inset in A, showing inflammatory cells incorporated with the fibrin network (x 2000).



**Figure 2.14:** One-day post-operatively at trauma site. (A): A stitch (arrow) coated with inflammatory cells (x 1000). (B): Dense, irregularly arranged connective tissue. Note the network of delicate fibrin threads (arrows) and coarse collagenous fibres (black arrow) spanning the trauma site and joining adjacent surfaces (x 1500).

underlying mesothelial layer was exposed (Figure 2.15 C). However, a similar finding was not detected on the visceral peritoneum. Mesothelial cells adjacent to the trauma site acquired different shapes: oval, satellite with elongated tapering or fish-like tails.

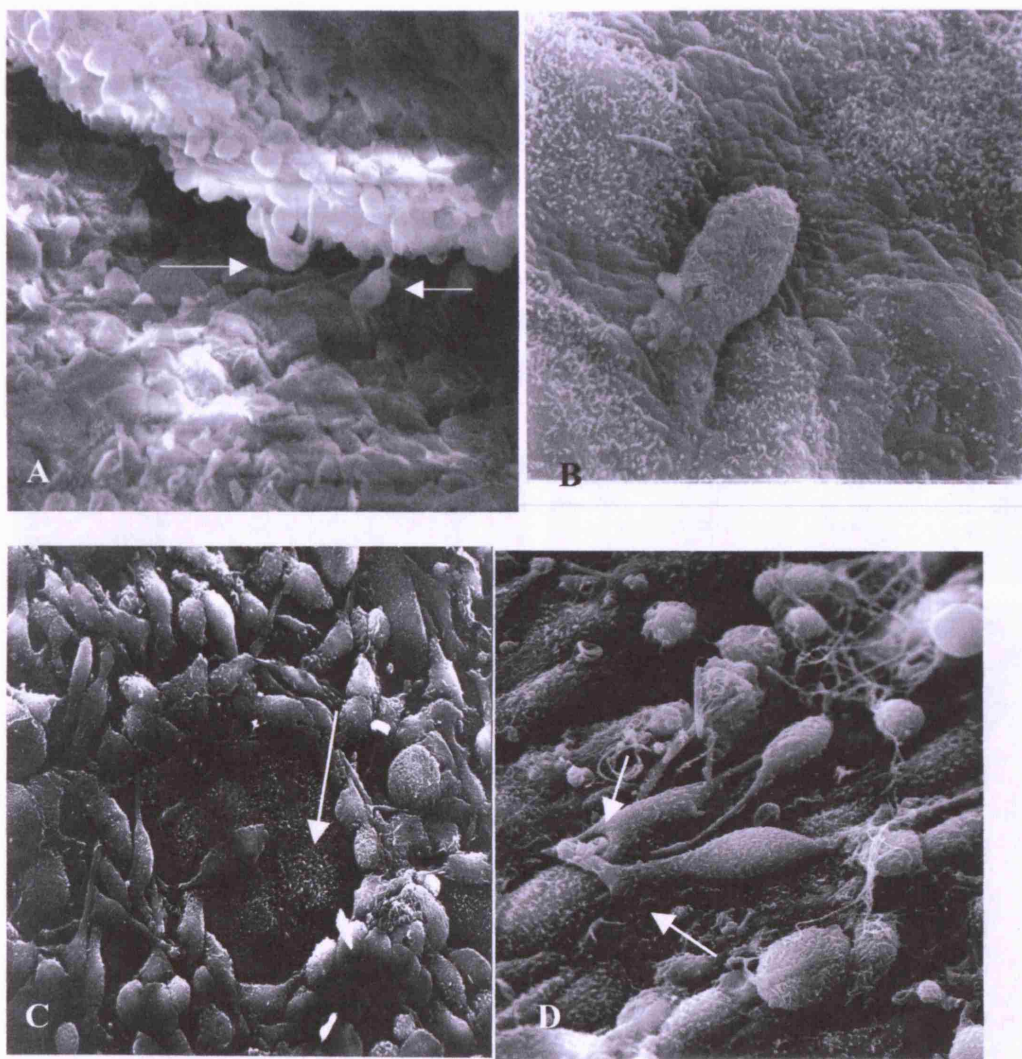
The bifid end of one mesothelial cell was seen in connection with either the body or the bifid end of another cell (Figure 2.15 D).

*Three days post-surgery*, the presence of fibrin at the site of trauma was less evident, but appeared more abundant at sites distant from the adhesion area. The visceral lining of the liver showed fibrin threads joining mesothelial cells together. Again there was a widening of the inter-cellular spaces indicating they were activated, although this area received no surgical trauma. The omentum was seen attached to the trauma site with multiple fenestrations that contained different cell types (Figure 2.16). Distant to the trauma site, activated mesothelial cells were seen as cuboidal in shape with multiple microvilli and widened inter-cellular spaces (Figure 2.17).

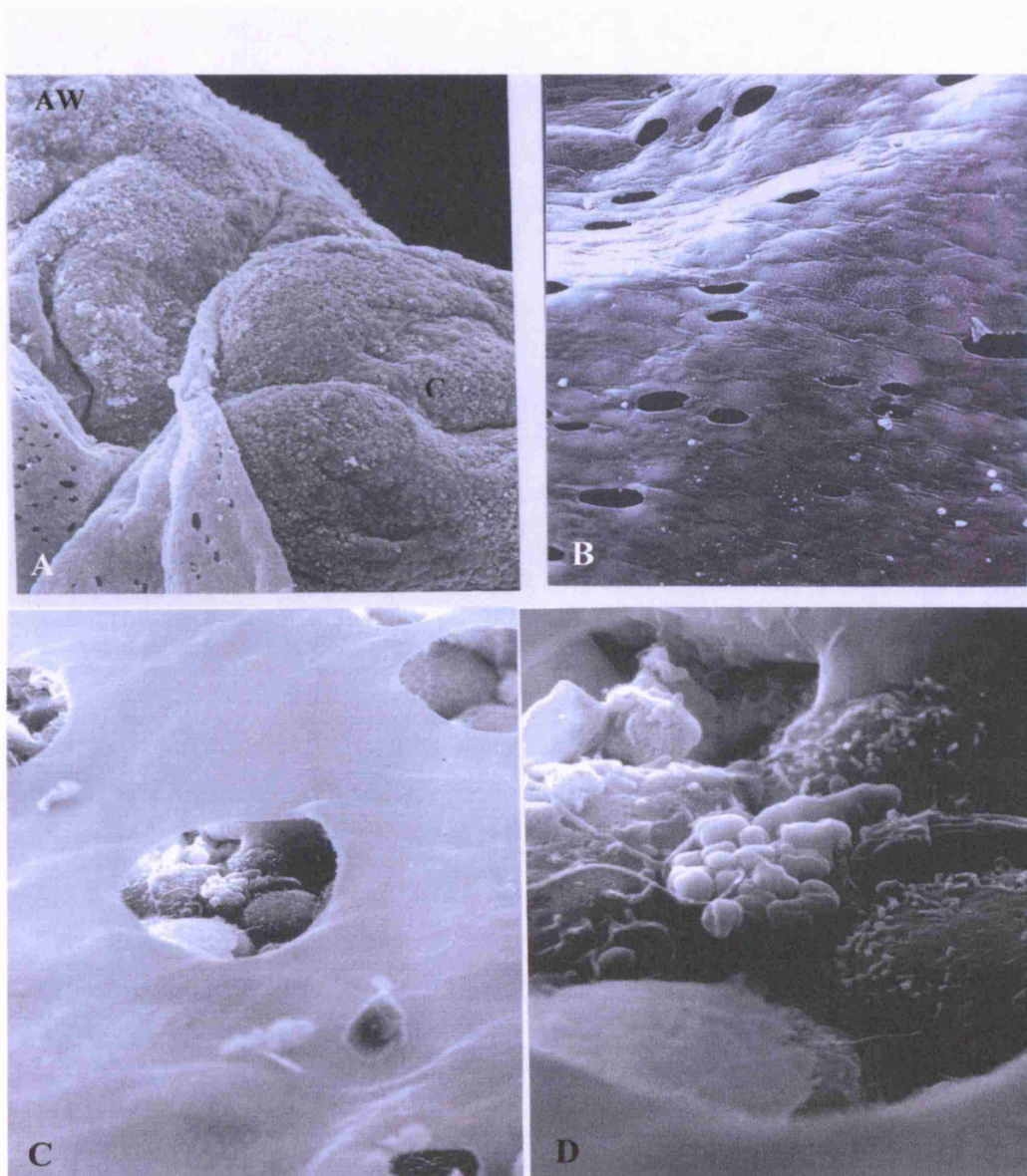
*Five-7 days post-surgery*, connective tissue was present between the two serosal surfaces while the initial fibrin fibres had disappeared. The appearance of the overlying serosal surface varied from area to area throughout the peritoneum on day 5. In some areas, the surface of the caecum was difficult to define from the body wall surface as mesothelial lining of the adhesion site was complete and continuous with the lining of the surrounding structures (Figure 2.18). In other areas defects were present on the surface layer with submesothelial layer exposed, but complete mesothelial covering of the surface of the adhesion had occurred by day 7. Some floating/migrating mesothelial cells were seen adjacent to the trauma site. These cells had multiple microvilli and the nucleus had a clear rim of chromatin with less prominent nucleoli.

*After 2 weeks- 6 months*, the adhesion tissue spanned the caecum and abdominal wall and was covered in mesothelial cells as at 7 days.



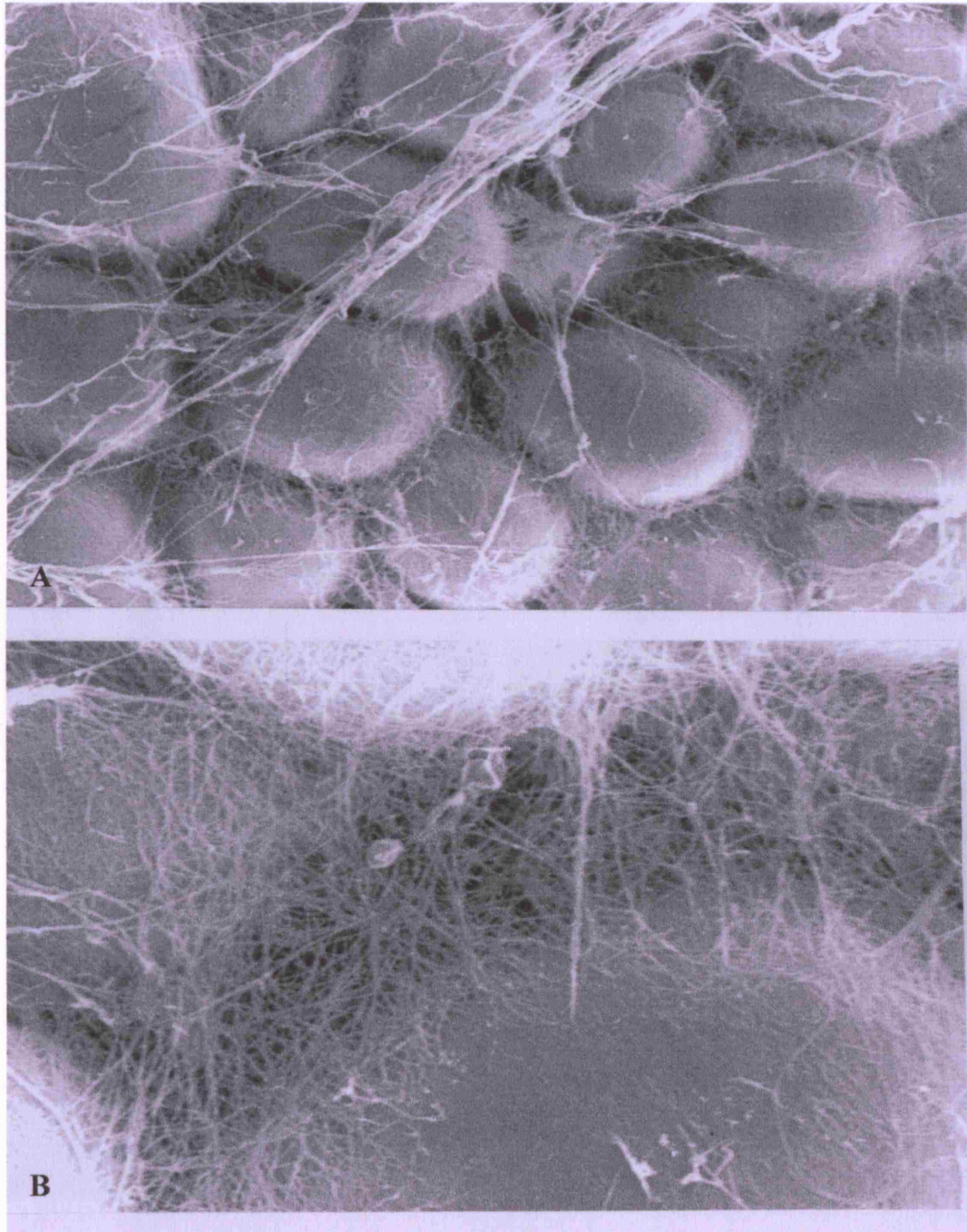


**Figure 2.15:** Two days post-operatively by scanning electron microscopy: (A) Two mesothelial cells (arrows) loosely attached to the surface lining (x500); (B) A mesothelial cell protrudes from the surface (x1500); (C) Clusters of inflammatory cells covering the parietal peritoneum leaving a circular area, where the microvilli of underlying mesothelial cells are seen (arrow) (x800); (D) Bifid-end of fibroblast-like cells (arrows) attached to other similar cells (x1500).

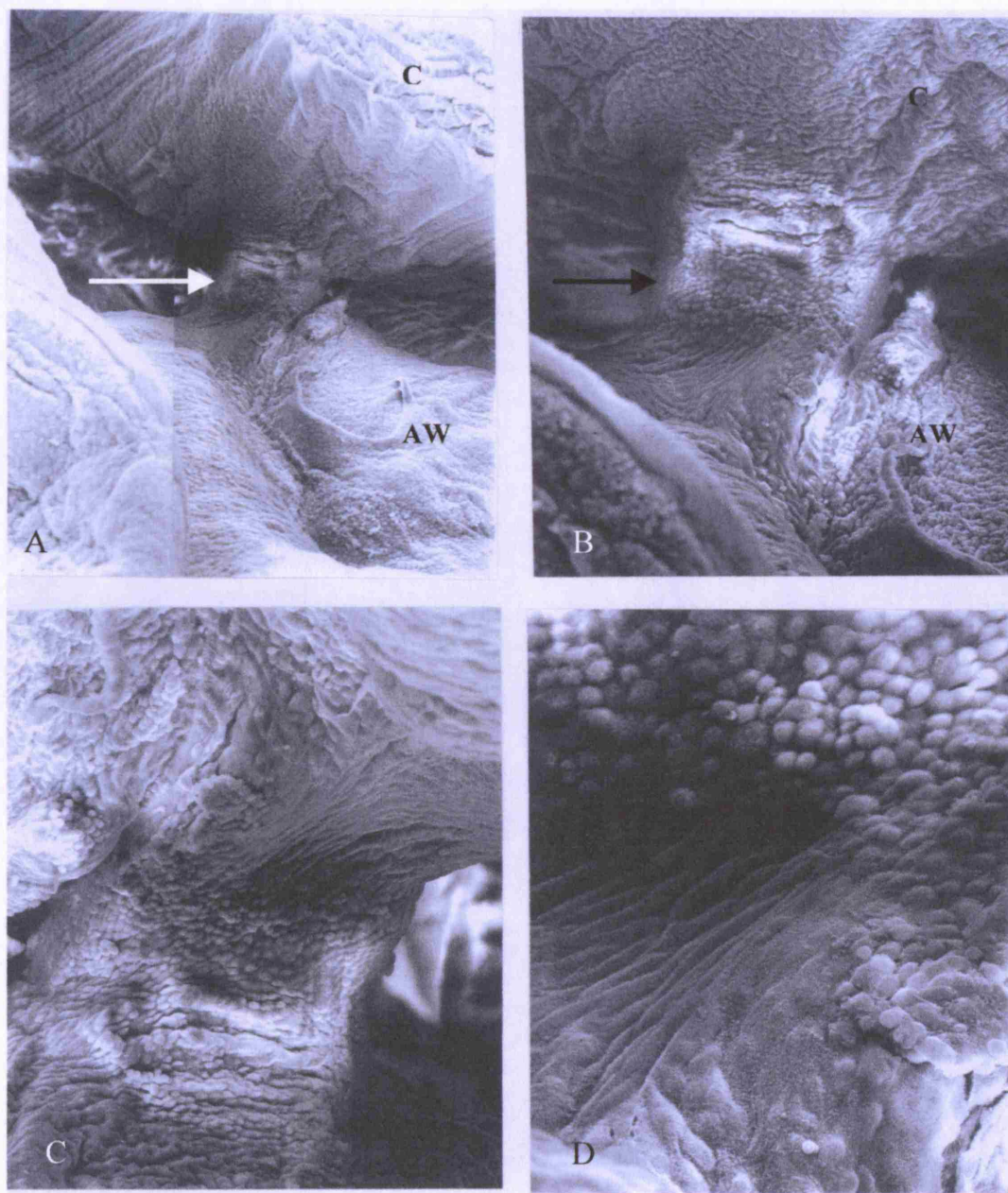


**Figure 2.16:** Three days post-operatively, omentum was attached to the trauma site between the caecum (C) and abdominal wall (AW) (x80) (B) Higher magnification of the omentum with multiple fenestrations (x400) (C) A fenestration showing different underlying cell types such as macrophages and red blood cells (x2000); (D) Higher magnification of (C) (x8000).





**Figure 2.17:** Three days post-operatively: (A) Mesothelial lining of the liver surface, away from the trauma site, showing fibrin threads (x600); (B) Higher magnification of the intercellular spaces showing fibrin fibres bridging the cells together (x2000).



**Figure 2.18:** Five days post-operatively by scanning electron microscopy: (A) Adhesion tissue (arrow) spanning between the caecum (C) and abdominal wall (AW) (x60). Sequential higher magnifications of A (B x100; C x150; D x400) showing mesothelial cells covering the adhesion continuous with the adjoining tissue.

## **2.4 DISCUSSION**

The present study monitored the formation of intra-peritoneal adhesions produced by a surgical experimental procedure. In humans, formation of adhesions is more or less inevitable following surgical trauma and is directly related to number of operative procedures undertaken (Weibel and Majno, 1973) with an incidence of 93% following abdominal surgery (Menzies and Ellis, 1990). The animal model developed was therefore based on a surgical trauma.

### **2.4.1 Animal Model**

It has been shown when designing our animal model that a single trauma was not sufficient for causing adhesions (Sulaiman, 1997, MSc thesis). As our aim was to simulate the circumstances and conditions of humans undergoing laparotomy, a multiple injury technique was used comprising of trauma to the caecum and abdominal wall, injury to a mesenteric vessel to cause bleeding and suturing of opposed damaged surfaces, limited to a specific area on the left side of abdomen, resulted in consistent reproducible standardized post-operative peritoneal adhesion being formed between caecum and abdominal wall in all the animals used. However, though the trauma was limited to one area, the effect was seen at distant sites including the liver. Approximation of damaged serosal surfaces by stitching was one of the main factors dictating the reproducibility of adhesion formation in our animal model. This procedure was lacking in many previous adhesion models and so may account for the problems of reproducibility (Milligan and Raftery, 1974). However, Ar'rajab and co-workers (1996) induced peritoneal adhesions by stripping a segment of parietal peritoneum in a rabbit and stitching the defect with non-absorbable suture material. In addition, Ricketts et al. (1999) induced adhesions by abrasion of a rabbit uterine horn and the adjacent parietal peritoneal surface, then approximating both surfaces by sutures. In both of these

models, suturing was an important component for the reproducible formation of peritoneal adhesion, so allowing them to be assessed over time.

#### **2.4.2 General histology and ultrastructural assessment of adhesion formation**

Few studies have investigated adhesion formation using more than one technique. In the present study, three different approaches were used to investigate adhesion formation; histology, scanning and transmission electron microscopy. Each method gave a different insight into adhesion formation. There were some difficulties in assessing the maturation of adhesion due to the need to interpret a dynamic process from a series of static appearances. Furthermore, at certain time points there was great variation in histological appearance from one area of the adhesion to another, also causing problems in assessment. In some of the adhesion specimens, there were areas showing patches of matrix with sparse cellularity, while other adjacent areas appeared fibrotic with excessive cellular infiltrate. Foreign body reaction to suture material was one of the reasons for the variation, but it is unclear what other factors may influence adhesion appearance.

In general, histological findings assessing the development of post-operative adhesions in our model were similar to other studies. Slade and Williamson (1968) suggested three phases of adhesion formation: first (0-7 hours) involved mesothelial cells desquamation, second (7 hours-10 days) associated with fibrin deposition and third (10 days-1 month) where collagen remodeling takes place. On the other hand, although similar phases were observed, Milligan and Raftery (1974) and Bakkum et al. (1995) suggested an earlier time scale regarding these processes in agreement with the current study. Ellis (1965) and Milligan and Raftery (1974) also found a fibrinous exudate at the trauma site in the first 24 hours associated with acute inflammatory cells mainly PMNLs and few lymphocytes. In this study a fibrin clot was present spanning the gap between the caecum and abdominal wall, 6 hours post-surgery, acting as a nidus for



adhesion formation. Other researchers (Ricketts et al., 1999) have reported the presence of tenacious fibrinous adhesions as early as one hour following injury, but this time point was not included in our study. Major changes occurred after 3 days with the disappearance of fibrin and presence of numerous macrophages followed by the appearance of blood vessels and fibroblasts as well as collagen deposition typical of granulation tissue. An interesting finding not reported by others was the splitting of the muscular layer of the caecal wall from the submucosa in 3 week adhesion specimens following tissue processing. This could be due to contraction of the regenerating muscle fibres or of the adhesion by myofibroblasts, causing gaping of surrounding tissue similar to skin contracture following wound healing. Hubbard and colleagues (1966), noticed regeneration of the muscle fibres of the anterior abdominal wall from day 4-6 onward, but contraction was not reported. Ultrastructural studies, TEM and SEM, provided a more detailed assessment of adhesion formation and allowed specific processes to be investigated further.

#### **2.4.3 Fat body and omental involvement in adhesion formation**

Omentum is known as “ the policeman of the abdomen “ because of its protective role in sealing off the inflammatory areas in the peritoneal cavity so minimizing their effects, as when an appendicular mass is formed following acute appendicitis. In previous studies, the omentum or pelvic fat bodies (PFBs) were reported to form adhesions as early as 24 hours post-operatively (Milligan and Raftery, 1974; Menzies and Ellis, 1990). This conforms with the findings in this study, but the role of the omentum in the initiation of adhesions and the changes that occur with remodelling have not been addressed. By using SEM, multiple fenestrations in the omentum were observed that incorporated a variety of inflammatory cells. These may represent the milky spots, areas of inflammatory cells overlying the lymphatics (Vanvugt, 1996). On day one post-injury, there was an infiltrate of inflammatory cells at

the attached area of the omentum and the trauma site. At a later stage, there was more collagen deposition in that area. Collagen deposition is a key process in adhesion formation and maturation as reported by Herrick et al. (2000). Whether adipocytes, mesothelial cells or fibroblasts play a major role in this process needs to be investigated further. Adherence of the omentum to site of injury within 24 hours post-surgery, and the presence of fenestrations studded with inflammatory cells suggest the omentum plays a role in adhesion formation in response to inflammatory insults in the peritoneal cavity as suggested by Wilkosz et al. (2005). Menzies and Ellis (1990) warned that the omentum is the organ most commonly involved in the development of adhesions and that surgeons should abandon the routine spread of omentum over damaged viscera to minimize or prevent adhesion formation. Indeed, Keubelbeck et al. (1998) found that omentectomy reduced adhesion formation in horses.

#### **2.4.4 Fibrin in adhesion formation**

Haemorrhage from any serosal surface is likely to lead to formation of fibrinous exudates. Following peritoneal trauma fibrinous exudate influences the initiation or resolution of adhesion formation. The mobility of intra-peritoneal viscera prevents the adherence of organs to each other, unless held closely. Fibrin provides an initial attachment between two serosal surfaces, but is overcome with lysis by fibrinolytic enzymes and therefore may not resist the forces of moving opposing organs (Vispond et al., 1994; van Goor et al., 1994). In this study, SEM showed the presence of fibrin exudate in the first 6 hours with multiple strands of fibrin spanning both serosal surfaces of caecum and abdominal wall. It is likely that this fibrin network act as a scaffold for migrating inflammatory cells that were found to be incorporated. Transmission electron microscopy showed the denuded surface lining at the trauma site with exposure of the submesothelial layer and deposition of fibrin, fragments of collagen, elastin and inflammatory cells. However, at a later stage, when cellular elements such as fibroblasts



and endothelial cells were present within the fibrin meshwork, collagen deposition and new blood vessels formation was observed. It is likely that the fibrin-rich adhesion undergoes organization resulting in permanent fibrous adhesion formation as suggested in other studies (Ryan, 1971; Abe et al., 1990; Haney, 1994). This critical time point occurred around day 3 post-surgery in our model and in other studies (DiZerga, 1992). Interestingly, fibrin fibres were also found coating mesothelial cells on surface of other organs such as liver. This suggests that the whole peritoneal cavity is affected by a localised injury and highlights that adhesions could also occur at sites distant from site of injury. The importance of the fibrinolytic system in deterring adhesion formation is analysed in Chapter 5.

#### **2.4.5 Mesothelial cell desquamation and re-epithelialisation**

Under the experimental conditions of the model, shedding of mesothelial cells appeared to be a critical event in the initiation of adhesion formation. Other studies suggest adhesion formation only occurs when two denuded surfaces were involved (Schade and Williamson, 1968; Haney, 2000). At the trauma site provisional adhesion at 6 hours post-operatively appeared by SEM as fibrin threads attached to both denuded serosal surfaces. Many inflammatory cells were embedded within the fibrin meshwork, but mesothelial cells were not seen as also reported by others (Menzies, 1992; Di Zerega, 2000). With TEM, fibrin was again evident on the traumatized denuded surface lining and there were no detectable mesothelial cells at the adhesion site up to day 3. However by day 5, the caecal and abdominal wall were clearly adherent and a mesothelial layer formed a continuous lining. At this stage, there was no evidence of any residual fibrinous debris in the peritoneal cavity.

Mesothelial cells detaching from distant sites, floating in peritoneal fluid and attaching to area of trauma are thought to be a pre-requisite for normal peritoneal healing as reported by Foley-Comer et al. (2002). In the current study, areas adjacent to

the trauma site had shown some mesothelial cells protruding from the surface lining, or other mesothelial cells appeared to be detaching or preparing to migrate during the first 2 days. Mesothelialisation of the surface of the developing adhesion occurred between days 3-5 post-surgery. These novel findings just highlight the complexity of mesothelial healing and the factors involved in promoting the healing process. Whether mesothelial cells are also present and are involved in ECM deposition within the adhesion is not known, but there is recent evidence to suggest they can undergo transdifferentiation (Young et al., 2000).

The presence of fibrin threads overlying the mesothelial surface of the liver three days post-operatively indicates that cuboidal mesothelial cells with abundant microvilli distant from the trauma site also appear to be activated. It seems there is a generalized response of the peritoneal cavity to injury possibly due to circulating factors in peritoneal fluid. This is usually a protective response by the body leading to neuro-endocrine secretions affecting target organs.

#### **2.4.6 Inflammation and adhesion formation**

The peritoneal cavity normally contains a small amount of serous fluid and cellular elements that consist mainly of macrophages, lymphocytes, polymorphnuclear neutrophils (PMNL) and mesothelial cells (Kubicka and Olszewski, 1989). Although not quantified in this study, the inflammatory cell profile appeared to follow a similar pattern to that described by Di Zerega (1994). An abundance of PMNLs in the initial hours post-injury was obvious at the trauma site where some were seen undergoing phagocytosis. This relates to the findings of Melnicoff and colleagues (1989) when they evaluated the kinetics of macrophage recruitment to the peritoneum after induction of acute inflammation. They observed that most of the labeled resident macrophages disappeared from the recoverable peritoneal cell population within the first hour and this coincided with the influx of PMNLs that was sustained for at least several days. In the

current study up to 2 days after injury, the most pronounced cellular elements at the trauma site were PMNLs. Between day 2 and 3, there was an influx of new cells, including macrophages, mesothelial cells and fibroblasts. These findings are similar to those of Rodgers and Di Zerega (1992), although they observed the influx of macrophages a day later, at day 3. When confronted by a peritoneal insult, inflammatory cells interact with one another via chemical signals. For example, cytokines secreted by PMNLs modulate macrophage function within 6-12 hours after surgery, and activated macrophages modulate the influx of PMNLs into the peritoneal cavity (Kuraoka et al., 1992; Cassettela, 1995).

Chung and colleagues (2002) reported an early influx of T cells lymphocytes following surgical trauma to the peritoneum with subsequent adhesion formation. It is well known that post-surgical macrophages play an important role in the repair of injured peritoneum. Enhancement of peritoneal macrophages by protease peptones markedly decreased postoperative adhesion formation (Ar'Rajab et al., 1995). They are also critical in the regulation of connective tissue matrix by secretion of cytokines, growth factors and connective tissue proteins, such as fibronectin, proteoglycans and proteases such as collagenase and elastase (Ryan et al., 1973; Rodgers & Di Zerega, 1992).

#### **2.4.7 Nerves and blood vessels in adhesions**

The detection of neuronal tissue by TEM on day 5 post-operatively at the trauma site within the adhesion tissue was surprising and unexpected at this early stage. The advanced development of neuronal tissue by 3 weeks was confirmed by the formation of myelin sheath with multiple axon fibres accompanied by excessive collagen deposition. A more detailed assessment of nerve growth in adhesions is addressed in Chapter 3. Blood vessels were first observed at day 3 and fully developed in the adhesions by day 5 displaying blood cellular elements within their lumens.

Angiogenesis is thought to contribute to the innervation process during normal tissue repair (Manek et al., 1993) and may guide the subsequent migration of axonal sprouts through the adhesion tissue. As vascular permeability factor (VPF)/vascular endothelial growth factor (VEGF) is thought to contribute to the development of adhesions therefore prevention of blood vessels formation by blocking an antibody to VEGF leads to less adhesion formation (Saltzman et al., 1996).

#### **2.4.8 Conclusion**

This study has generated new information to increase understanding the pathophysiology and mechanisms involved in post-operative adhesion formation. By developing the animal model, a certain level of localised trauma was needed to induce adhesion formation and the effect was seen at distant sites at later stages. Using a combination of techniques highlighted some unexplored processes during post-operative adhesion formation.

Histologically, the occurrence of contraction of the adhesion tissue at 3 weeks post-surgery was a novel finding that has not been reported before. By SEM, the meshwork of fibrin threads at the trauma site with the incorporation of inflammatory cells in association with protruding or possibly detaching mesothelial cells in the first 2 days post-operatively, highlighted the initial stages in the healing process. The presence of abundant inflammatory cells surrounding the suture material, demonstrated the intensity of the inflammatory response and variations in the structure of adhesion due to a foreign body reaction. The formation of omental adhesions at day 3, with the presence of omental fenestrations associated with inflammatory cells has not been documented in previous adhesion formation studies. The abundance of fibrin threads overlying the mesothelial lining of the liver surface distant to the trauma site on day 3 post-surgery indicates universal mesothelial cells activation and a possible response to circulating or peritoneal fluid factors.

By using TEM, the abundance of fibrin at the trauma site in association with fragments of elastin, collagen and inflammatory cells was noted. The finding of nerve fibres in post-operative peritoneal adhesions in an experimental model was novel and was the motive to pursue the growth of nerves in adhesions further Chapters 3 and 4.

### **CHAPTER THREE**

## **GROWTH OF NERVE FIBRES INTO MURINE PERITONEAL ADHESIONS**

### 3.1 INTRODUCTION

Chronic abdominal/pelvic pain of more than six months duration is a common post-operative complaint (Howard, 1994; Keltz et al., 1995; Popora and Gomel, 1997). Peritoneal adhesions develop in 67-93% of patients after laparotomy (Luijendijk, 1996). This has led to the concept that adhesions are the cause (Kresch et al., 1984; Trimbos et al., 1990; Stout et al., 1991) and adhesiolysis is the therapeutic approach of choice. This concept has been controversial. Some clinicians question the role of adhesions as the cause of chronic abdominal/pelvic pain (Rapkins, 1986; Alexander-Williams, 1987) and many consider adhesions as structureless scar tissue (Ellis, 1962) although few experimental studies have suggested that adhesions are involved in pain sensation (Almeida and Val-Gallas, 1997). Sutton and McDonald (1990) performed laser laparoscopic adhesiolysis in 65 patients with chronic pelvic pain and achieved symptomatic relief in 84% of cases with a follow up of 1-5 years. Steege and Stout (1991) and Fayez et al. (1994) reported that up to 88% of patients were relieved of chronic abdominal pain following adhesiolysis. To understand these observations, a brief review of nerve fibres present in the peritoneum needs to be described.

The peritoneum is formed of parietal and visceral layers. The parietal peritoneum is innervated by somatic nerves (T5-L2), which it shares with the body wall (Moore, 2002) whereas the somatic nervous system is formed of sensory nerves that conduct touch, pressure, pain and temperature sensations to the central nervous system (CNS) and motor nerves that conduct a response from the CNS to the peritoneum to cause muscles to contract. The parietal peritoneum is rich with pain receptors that conduct pain stimuli from the peritoneal cavity to the CNS. Conversely the nerve supply of the visceral peritoneum is derived from the autonomic nervous system and the enteric nervous system which is a separate division of the autonomic nervous system called “the enteric nervous system” (Gabella, 1979; Gonella et al., 1987). The autonomic

nervous system (ANS), which is also known as the involuntary or visceral nervous system, controls involuntary activity such as the action of the heart, movements of the viscera, the state of the blood vessels and the secretion of glands. It includes a 'sympathetic' and a 'parasympathetic' division. Commonly one system is excitatory and the other inhibitory. The visceral peritoneum is devoid of pain receptors and many clinicians are aware that handling the bowel during surgery is usually painless in procedures performed under local anaesthesia. However, it is supplied with stretch receptors that conduct stretch or distension sensations to the CNS. Patients with hepatomegaly or splenomegaly (enlargement of the liver or spleen respectively) feel heaviness, discomfort and occasional pain at the site of the enlarged organ due to stretching of the overlying visceral peritoneum that encased these organs and is anatomically known as the capsule of liver or spleen.

It has been reported that human pelvic adhesions contain nerve fibres (Kligman et al., 1993; Tulandi et al., 1998) whose origin and function has not been defined. The aim of this study was to assess the temporal and spatial pattern of nerve growth in an experimental surgical model of adhesion formation. Since studies examining nerve growth in human adhesion tissues are not feasible, our murine adhesion model was used which allows the standardization of surgical procedures, anatomical sites and pathological conditions. In addition, specimens can be examined at fixed time points during adhesion maturation and parallel studies can be performed in a reproducible manner. The growth of nerve fibres has not been previously assessed in an experimental adhesion model. Nerve fibres were characterized by immunocytochemistry using antibodies against specific nerve markers such as a protein of synaptic vesicles (synaptophysin), as well as specific neuropeptides such as calcitonin gene related peptide (CGRP) expressed by sensory nerves and substance P present in pain conducting sensory nerves (Gibbins, 1987). Vasoactive intestinal peptide (VIP) a



marker for the enteric nervous system, and tyrosine hydroxylase (TH), an intermediary enzyme in catecholamine synthesis, for the autonomic nervous system were also immunolocalized. Acetylcholinesterase whole-mount histochemistry was performed to detect the 3-dimensional arrangement of small nerve bundles and associated blood vessels between adhesion and adjacent structures. The presence of myelinated and non-myelinated axons was determined ultra-structurally by transmission electron microscopy.

## **3.2 MATERIALS AND METHODS**

All chemicals were obtained from Sigma (Poole, Dorset, UK) or BDH (Lutterworth, UK) unless otherwise stated.

### **3.2.1 Animals**

Home Office licence requirements were fulfilled for the project and personal use. Forty-two male Balb-c mice, 8-10 weeks old with average weight of 30 grams were used throughout this work (Harlan Ltd., Bicester, UK).

### **3.2.2 Operative procedure**

Mice ( $n = 42$ ) were operated under general anaesthesia induced with 4% halothane and maintained with 1.5-2% delivered with a mixture of nitrous oxide: 1.5L/min and oxygen: 3L/min. All surgical procedures were performed under aseptic conditions as described previously (Chapter 2).

### **3.2.3 Perfusion**

Groups of six mice were given a terminal anaesthesia at one to eight weeks post-operatively by an intra-peritoneal (I.P.) administration of 0.1 ml of Hypnorm (fentanyl citrate 0.315mg/ml and fluanisone 10 mg/ml; Janssen Pharmaceuticals, Grove, Oxford, UK) and 0.1 ml of Hypnovel (5mg/ml; Roche, Switzerland). Vascular perfusion was performed by initially injecting 20 ml Phosphate Buffered Saline (PBS) pH 7.4, containing 0.1 mg Sodium nitrate and 1 ml of Heparin (1000 i.u. /1 ml; C P Pharmaceutical, Wrexham, UK) through a cannula inserted into the left ventricle of the heart allowing drainage of the blood through a hole in the right atrium. The appropriate fixative was then administered using the same route as described. The peritoneal cavity was opened by reflecting to the left a square-shaped flap of the abdominal wall. Assessment of the whole viscera was performed. Adhesions (fixed in situ) involving

part of the caecum with the body wall were removed. The caecum was opened along its anti-mesenteric border away from the adhesion and the contents were washed away gently with distilled water. Tissues were then processed for immunocytochemical (n = 2), histochemical (n = 2) and ultra-structural examination (n = 2) as discussed below. A group of six uninjured mice acted as controls.

#### **3.2.4 Immunohistochemistry**

Animals were perfused with 50 ml of 4% paraformaldehyde (4%PF) in PBS pH 7.4. The excised adhesion specimen was further fixed in 4%PF in PBS for one hour at room temperature. Specimens were then washed in PBS for 30 minutes, transferred to 7% Sucrose with 0.1% Sodium azide ( $\text{NaN}_3$ ) in PBS, pH 7.2 and stored overnight at 4°C. Specimens were embedded in optimal cutting compound (OCT; Miles Inc., USA) and frozen over liquid nitrogen. Thirty to forty frozen sections (10µm) per block were collected on 0.01% poly-L-lysine coated slides (Sigma, Poole, UK) and stored at -20°C until used. Five to ten sections were selected approximately 50-100µm apart and incubated overnight with well-characterized polyclonal primary antibodies in a humidified chamber at room temperature. Antibodies used included those against CGRP (Rabbit anti-rat alpha calcitonin gene-related protein; Affiniti, Exeter, UK; batch Z01172); synaptophysin (Rabbit anti-synthetic human synaptophysin, DAKO, Glostrup, Denmark; batch 073/502); substance P (Rabbit anti-human substance P, Chemicon International, Amersham, UK); vasoactive intestinal peptide (Rabbit anti-rat VIP; Chemicon International, Amersham, UK); tyrosine hydroxylase (Rabbit anti-rat TH; Chemicon International, Amersham, UK). Certain sections were also immunostained for blood vessels using primary antibody against von Willebrand factor (vWF; Sheep anti-mouse; Chemicon International, Amersham, UK). All primary antibodies used were at a dilution of 1:1000 after performing optimisation studies. Control sections were

incubated with either normal blocking serum (Donkey normal serum or goat normal serum; Sigma, Poole, UK) or rabbit IgG (Sigma, Poole, UK) instead of primary antisera. A few sections within each block were also stained with haematoxylin & eosin (H & E) for tissue orientation.

After washing 3x in PBS for 30 minutes, all sections were incubated for one hour at room temperature with a 1:250 dilution of biotinylated donkey anti-rabbit IgG (Amersham, Buckinghamshire, UK). Sections were then washed 3x in PBS for 45 minutes before they were incubated with streptavidin-fluorescein reagent (Amersham, Buckinghamshire, UK) diluted 1:100 for 1 hour at room temperature. Following a final wash in PBS, the sections were mounted in Citifluor (Citifluor, London, UK), coverslipped and examined under a microscope with fluorescent filters (Axiophot microscope, Zeiss, Germany) and photographed within a month with PROVIA Fujichrome 1600 ASA film.

### **3.2.5 Acetylcholinesterase histochemistry**

Acetylcholinesterase histochemistry was performed according to the method of Baker et al. (1986). Animals were perfused with 50 ml of cold 10% buffered formalin, pH 7.2 as the fixative. Excised adhesion tissue was pinned to sylgard (Dow-Corning, Weisbaden, Germany) as a whole mount preparation and further fixed for one hour with 10% buffered formalin at 4°C. Specimens were then washed and immersed in 100 ml PBS pH 7.2, containing 1280 units of hyaluronidase (Sigma, Poole, UK) and tetraisopropylpyrophosphoramidate (iso-OMPA; final concentration  $10^{-4}$  M; Sigma, Poole, UK) overnight at 4°C. Tissue was then incubated with acetylcholine incubating solution (5mg acetylcholine iodide, 6.5ml of 0.1 M acetate buffer with 1.5% Triton X-100, 0.5 ml of 0.15M sodium citrate, 1ml of 30 mM copper sulphate, 10ml of 1mM iso-OMPA and 1ml of 5mM potassium ferric cyanide) for 24 hours with the solution being changed every 2 hours for 8 hours and then every 8 hours at room temperature.

Specimens were regularly viewed (approximately once every hour) through a dissecting microscope (Zeiss, Germany). When nerve staining was sufficiently intense the reaction was stopped. The specimen was immersed in distilled water for one hour then fixed again in 10% buffered formalin, mounted in 70% glycerol between glass slides and visualised through a stereo dissecting-microscope (Zeiss, Germany) using epi-illumination supplied by either a circular light source or twin fibroptic light. Photographs were taken using a Nikon camera and Ilford black and white HP5 film rated at 400 ASA.

### **3.2.6 Transmission Electron Microscopy**

Animals were perfused with 50-60 ml fixative solution of 1% paraformaldehyde and 5% glutaraldehyde in 100 mM sodium cacodylate buffer at pH 7.4. The perfusion time was around 2 minutes. The peritoneal cavity was opened, the adhesion specimen excised and further fixed overnight in perfusion solution at room temperature. The specimen was then pinned out on a Sylgard plate under a Nikon dissecting microscope (Japan) to isolate the area of interest. Tissue was washed in fresh cacodylate buffer and post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer pH 7.4 for 1 hour. Specimens were washed in sodium cacodylate buffer, stained in an aqueous saturated solution of uranyl acetate for 1 hour and after a brief wash in distilled water were dehydrated in graded alcohols ranging from 50% ethanol to absolute ethanol and then embedded in Araldite epoxy resin. Epoxy resin (Araldite<sup>TM</sup>) was prepared by mixing together 51g of CY212 polymer with 49g of dodecenyl succinic anhydride (DDSA) polymer and heated to 65°C. One ml of benzyl dimethyl amine (BDMA) and 1.5ml of dibutyl-phthalate hardeners were added and the resin mixed thoroughly. Specimens were put into propylene oxide followed by a 50:50 resin/propylene oxide solution and finally infiltrated overnight in resin. The resin containing the specimens was poured into shallow foil dishes which were placed in an oven at 70°C to harden.

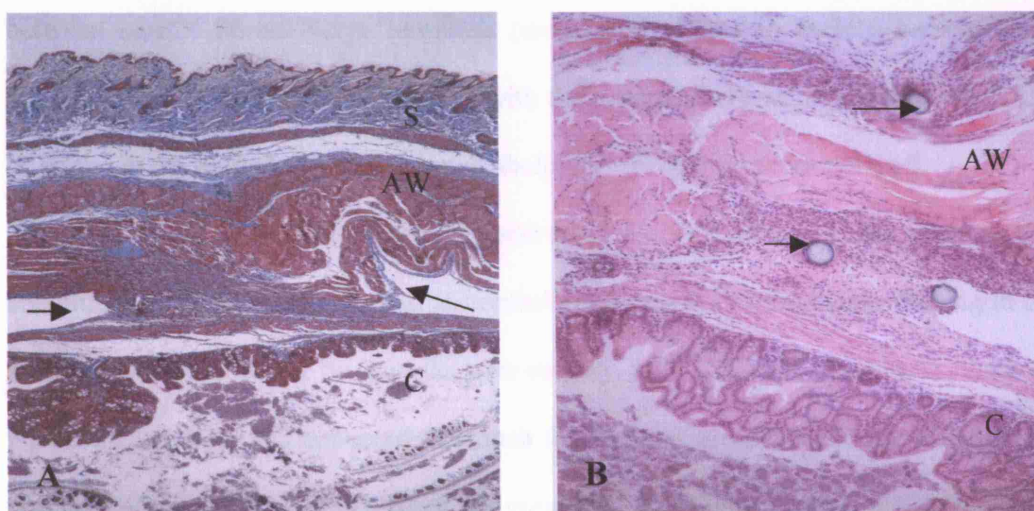
After three days, the individual tissue was separated into smaller blocks ready for sectioning.

**For light microscopy:** sections were cut with dry glass knives on a Reichert microtome (Austria) at a thickness of 0.5-1 $\mu$ m. For the thinner sections a wet knife was used. Sections were floated onto drops of distilled water on glass slides and water was evaporated on a hot plate at 50°C. Sections were then stained for 30-90 seconds (according to thickness) with 1% alcoholic toluidine blue (Sigma, Poole, UK). Excess stain was removed with 50% ethanol which also served to bring about staining contrast. Slides were dried on a hot plate and mounted with a drop of Araldite<sup>TM</sup> resin under a cover-slip. Sections were examined with Zeiss Axiophot light microscope using phase contrast optics. Photographs were taken with Kodak Ektachrome 64 tungsten colour reversal film.

**For transmission electron microscopy:** Sections (70-90nm) were cut with a wet glass knife and collected onto copper grids. Further staining with 3% uranyl acetate in 50% alcohol as well as lead citrate was used to give the sections increased contrast for electron microscopy. All staining solutions were filtered using Acrodisc 32 syringe filter 0.2 $\mu$ m (Gelman Sciences, Ann Harbor, USA). Copper grids were floated section-side down on uranyl acetate (3% in 50% ethanol) for 4 minutes in a sealed container in the dark. The grids were then thoroughly washed in distilled water and floated again sections-side down on a drop of lead citrate on Nescofilm (Nippon Shoji Kaishi, Japan) surrounded by sodium hydroxide pellets and contained in a sealed dish. The grids were left on the lead citrate drops for two minutes after which they were rinsed for ten seconds in four 10 ml vials of clean distilled water. When dried the sections were viewed and photographed in a Philips 400 transmission electron microscope (Eindhoven, Holland) using Kodak electron microscope 4489 film.

### 3.3 RESULTS

All animals in this study developed fibrous post-operative peritoneal adhesions between the opposing injured serosal surfaces of the caecum and abdominal wall within one-week from surgery (Figure 3.1A). At this stage the margins of the adhesions were covered by a continuous layer of mesothelial cells and contained abundant collagen fibres and an extensive distribution of blood vessels including arterioles, venules and capillaries. Inflammation was prominent in the early stages (1- 2 weeks) but later was confined to the sites of the stitches (4 weeks) (Figure 3.1B). There were no post-operative infections or deaths in any of the animals. Control animals developed no adhesions.



**Figure 3.1:** Post-operative peritoneal adhesions. (A): 1 week post-operatively showing peritoneal adhesion (arrows) inbetween the caecum and abdominal wall (Masson's trichrome, x 300); (B): 4 weeks post-surgery, with inflammatory cells surrounding the stitches (arrows) (Haematoxylin & Eosin, x 430). S: Skin; AW: abdominal wall; C: caecum.

#### 3.3.1 Synaptophysin Immunohistochemistry

In control un-operated mice synaptophysin-immunoreactive axons (SYN-IR) were present in the submucosa of the caecum forming an elaborate meshwork of nerves

(submucosal plexus) and lying parallel to the mucosal surface (Figure 3.2). This network included ganglia with branching axons, either single axons with a distinct beaded or varicose appearance or bundles of axons. In addition, the myenteric plexus in the muscle layer of the caecum demonstrated SYN-IR branching ganglia and numerous fibres as well as axons lying parallel to each other within muscle bundles. Positively stained nerve plexuses were also found in the adventitia of arteries and arterioles as elongated networks. Veins were associated with positive axons but fewer in the arteries. In contrast to the mucosa, the serosa and subserosa (mesothelial and submesothelial) layers were devoid of SYN-IR axons. In the abdominal wall SYN-IR axons were less abundant than in the caecal wall and were mainly found in neuro-vascular bundles between muscle fibres. Nerve terminals (motor end plates) on skeletal muscle fibres stained intensely for synaptophysin. As with the caecum there were no detectable SYN-IR axons in the mesothelial and submesothelial layers of the abdominal wall.

A week after injury the distribution of SYN-IR axons in the caecum and abdominal wall was similar to that in control animals with no detectable staining in the adhesion tissue. However by 2 weeks post-surgery, a few SYN-IR fibres were observed within the adhesion and appeared to branch from nerves present in the subserosa of the caecal wall. From the third week (Figure 3.3 A) multiple short SYN-IR axons originating from the myenteric plexus with varicosities were found beneath the thickened serosal layer parallel to the caecal surface at sites adjacent to the site of adhesion. At this stage few nerve fibres were found originating from the abdominal wall. The findings at week 4 were similar (Figure 3.3 B) but by 6 weeks post-surgery numerous SYN-IR branching axons with varicosities were observed within the adhesion originating from the caecal wall as well as the abdominal wall. Eight weeks after injury, multiple branching axons appeared to traverse the adhesion originating from both the abdominal wall and the caecum (Figure 3.3C).

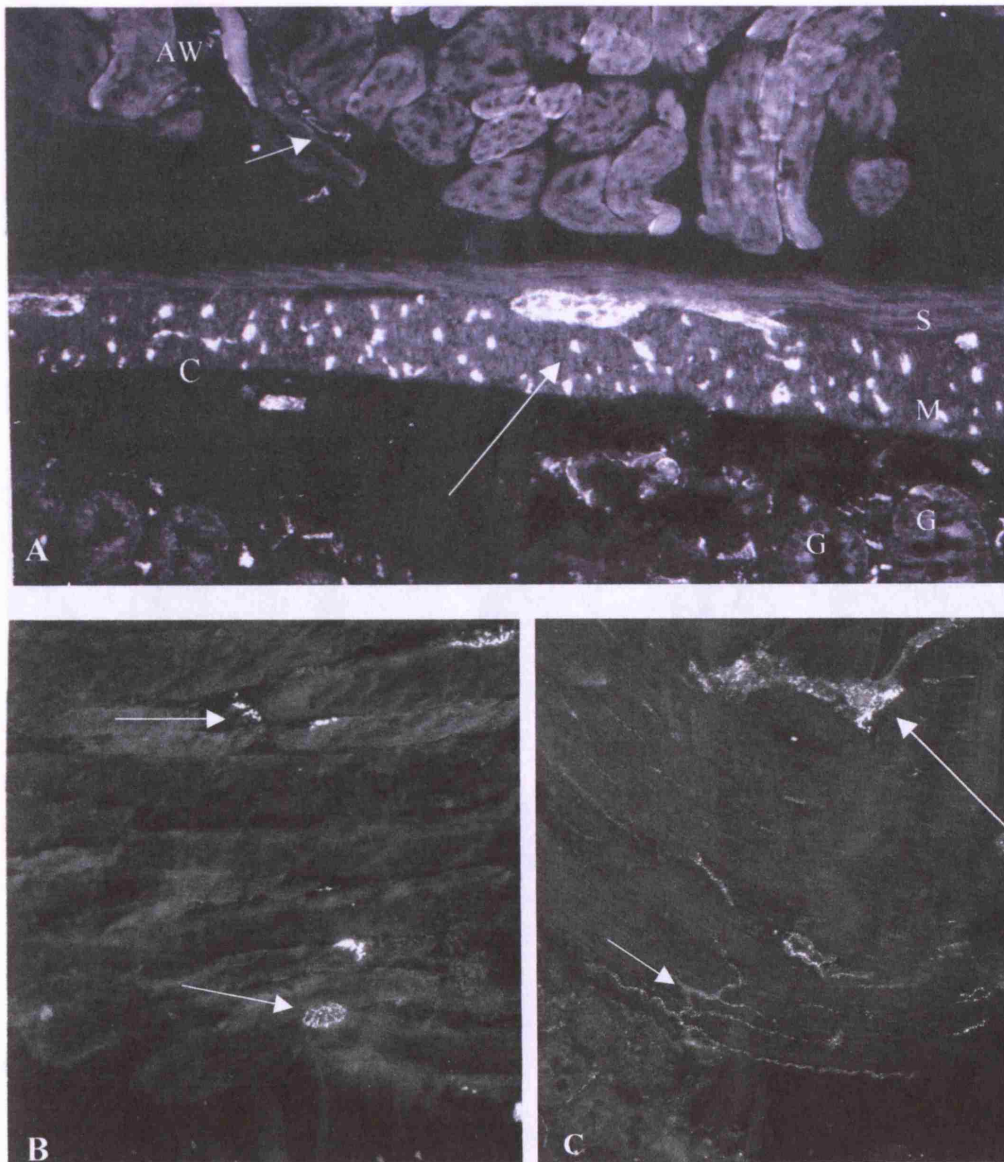


### **3.3.2 Calcitonin Gene Related Peptide (CGRP) Immunohistochemistry**

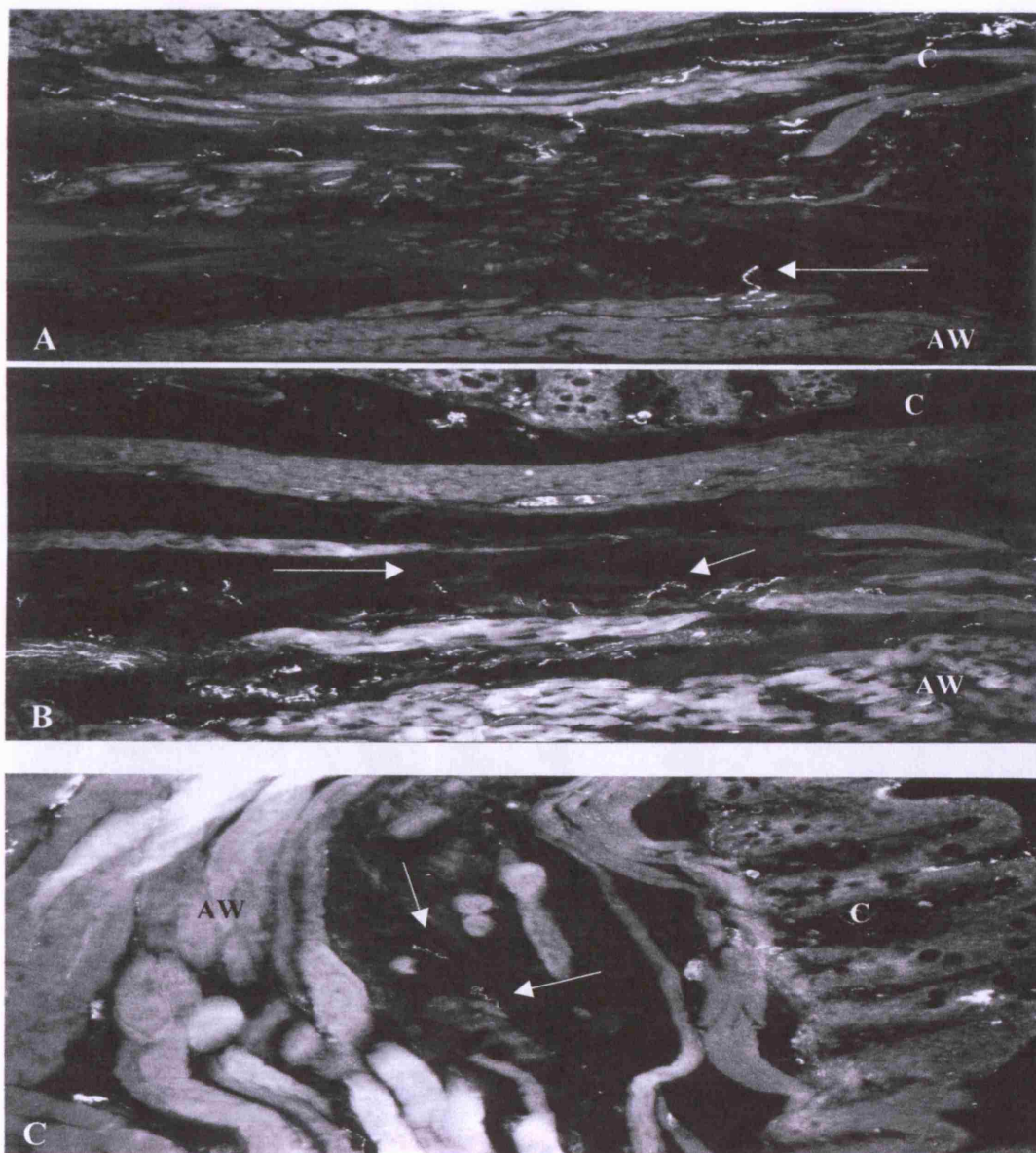
The distribution of calcitonin gene related peptide immunoreactive (CGRP-IR) axons in both control tissue and peritoneal adhesions were similar to that observed with synaptophysin immunostaining (Figure 3.4A). At two weeks post-surgery short CGRP-IR fibres were observed arising from the caecal subserosa (Figure 3.4B). By the third week numerous immunofluorescent axons, some with multiple varicosities were found within the adhesion tissue originating mainly from the caecal, but also the abdominal wall (Figure 3.4C). By 6-8 weeks long tortuous CGRP-IR axons traversed the adhesion; branching from both the caecum and the abdominal wall (Figure 3.4D). Interestingly, a further adhesion site distant to the site of trauma was found joining two opposing loops of small bowel in one of the animals and this was also processed for CGRP immunohistochemistry (Figure 3.5). The axons of the branching ganglia of each myenteric plexus had traversed the subserosal and the serosal layers of each loop of bowel and traversed the adhesion site so that the distinction of one plexus from the other was not clear.

### **3.3.3 Substance-P (S-P)**

The distribution of substance-P immunoreactive (SP-IR) nerve fibres in both control and adhesions was similar to that of SYN-IR and CGRP-IR fibres. By 2 weeks post-surgery short parallel SP-IR fibres branched from the myenteric plexus of the caecal wall towards the centre of adhesion (Figure 3.6A). By 3 weeks varicose SP-IR axons were seen within the adhesion, originating from both the caecum and the abdominal wall (Figure 3.6B) and at 8 weeks both long and short tortuous SP-IR axons were observed (Figure 3.6C).

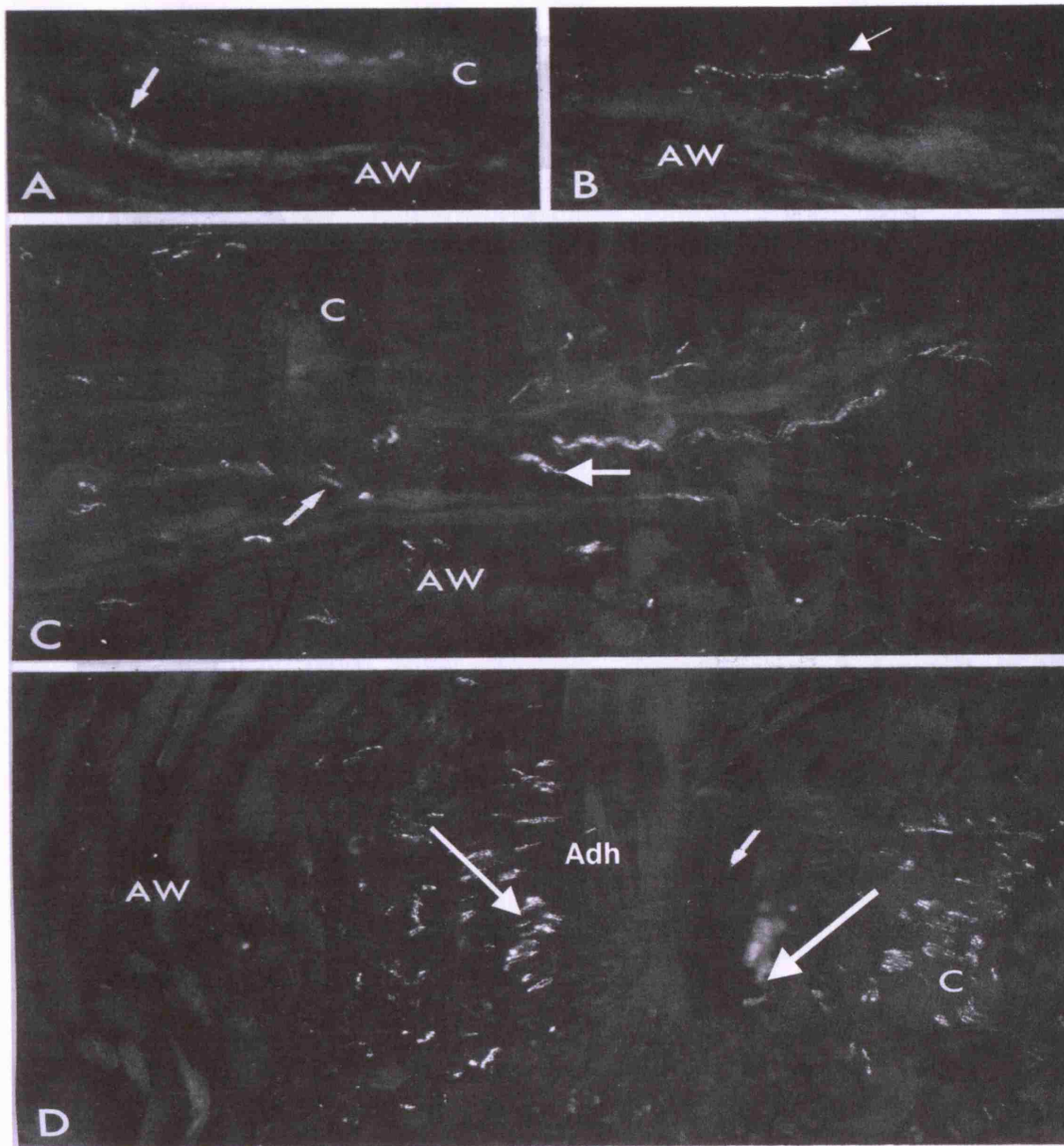


**Figure 3.2:** Control tissue (A): Abdominal wall showing synaptophysin immunoreactive (SYN-IR) axons at neuromuscular junction sites (short arrow) and caecum with multiple SYN-IR ganglia (long arrow) (x720). (B): Neuromuscular junction sites present in the abdominal wall (arrows) (x1470). (C) Multiple ganglia (long arrow) with branching nerve fibres (short arrows) from both nervous plexuses of the caecal wall (x1470). C: caecum; AW: abdominal wall; S: serosal layer of caecum; M: muscular layer of caecum; G: glands and mucosal layer of caecum.

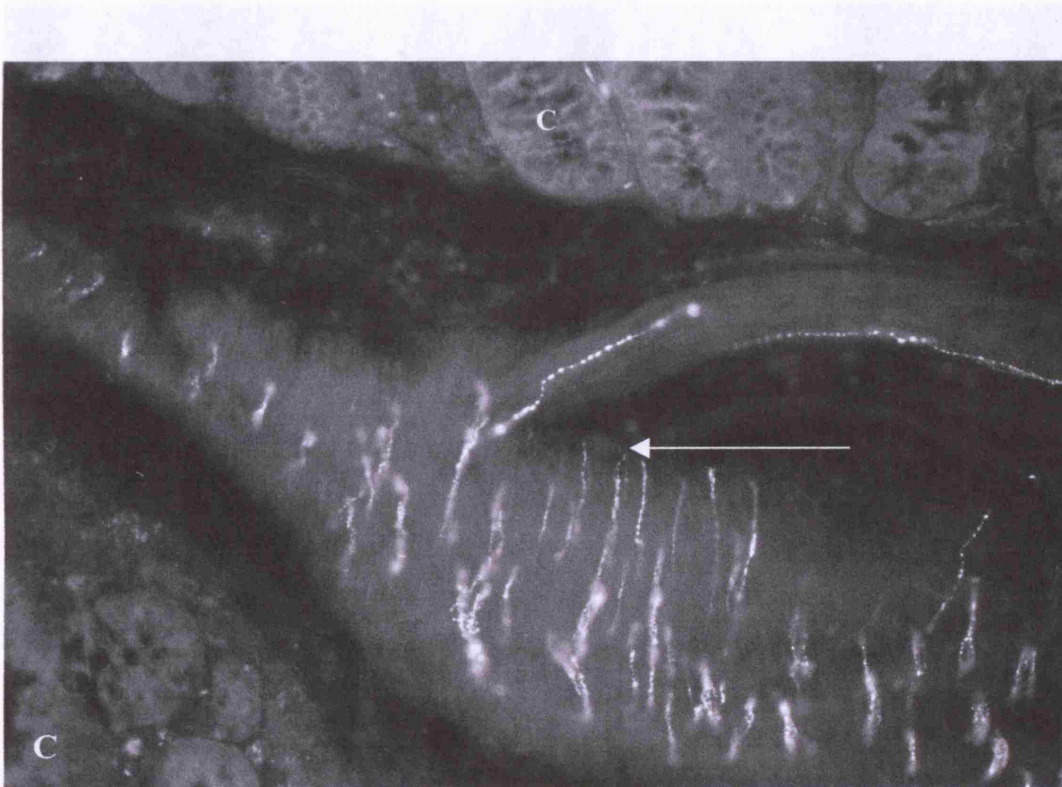


**Figure 3.3:** Synaptophysin immunoreactive (SYN-IR) nerve fibres observed in post-operative peritoneal adhesions. (A) At 3 weeks post-surgery, showing tortuous nerves with varicosities from both caecal and abdominal wall (arrows). (B) Multiple nerve fibres with varicosities found in adhesion tissue 4 weeks post-operatively (arrows). (C) At 8 weeks post-surgery, nerve fibres were found in the centre of the adhesion tissue (arrows) between the caecum, (C), and abdominal wall, (AW).

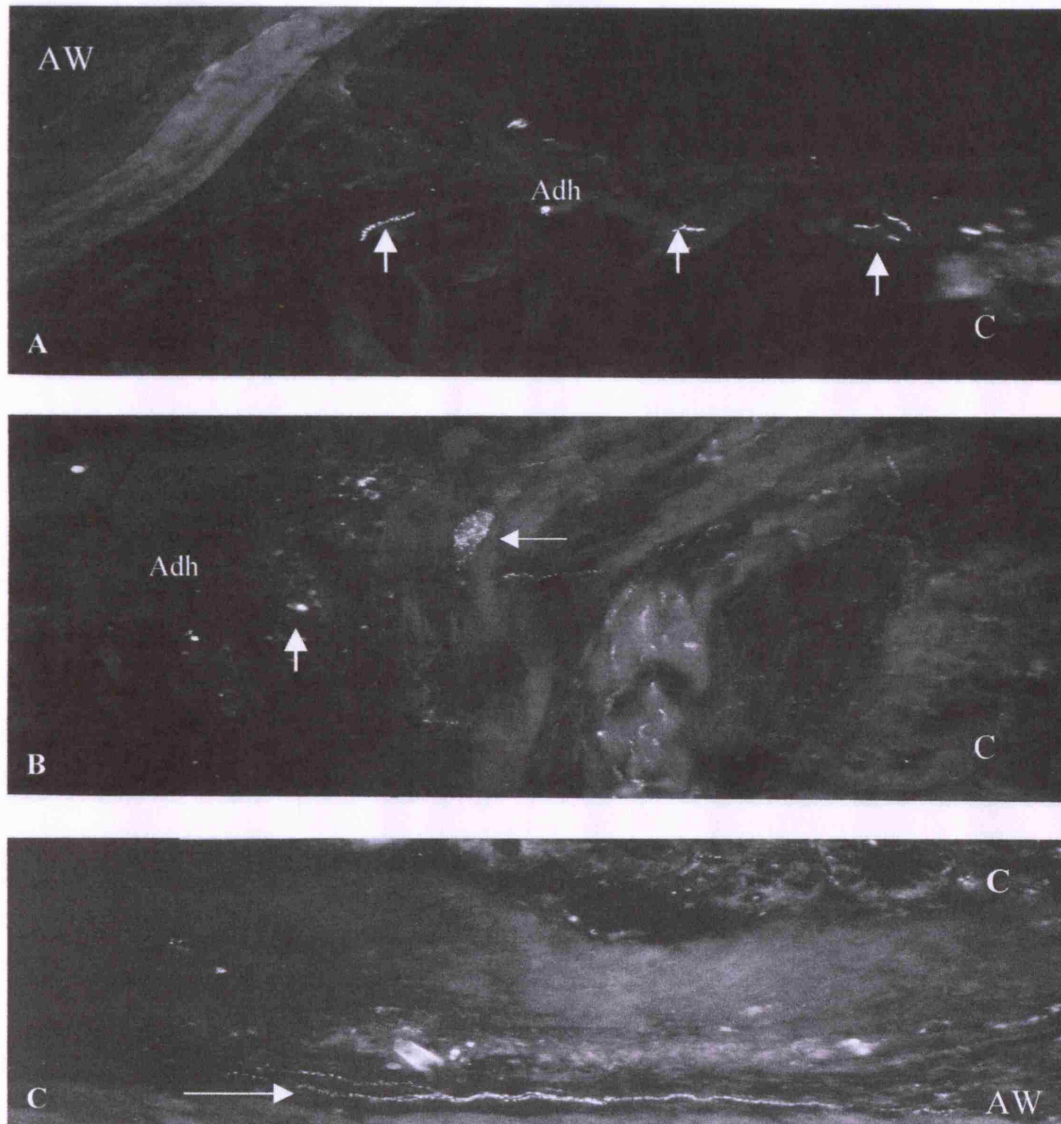




**Figure 3.4:** Calcitonin gene related peptide immunoreactive (CGRP-IR) nerve fibres in adhesions. (A): Peritoneal adhesion at 2 weeks post-surgery demonstrating two varicose nerve fibres (arrow) traversing between the caecum (C) and abdominal wall (AW) (x1470). (B): Three weeks post-surgery (x1470), and (C): at four weeks post-surgery with multiple nerve fibres in the centre of the adhesion (arrows) (x1250). (D) Peritoneal adhesions at 6 weeks post-surgery with multiple nerve fibres (long arrows) surrounding the site of suture material at the adhesion site (short arrow) (x 720). Adh ; adhesion

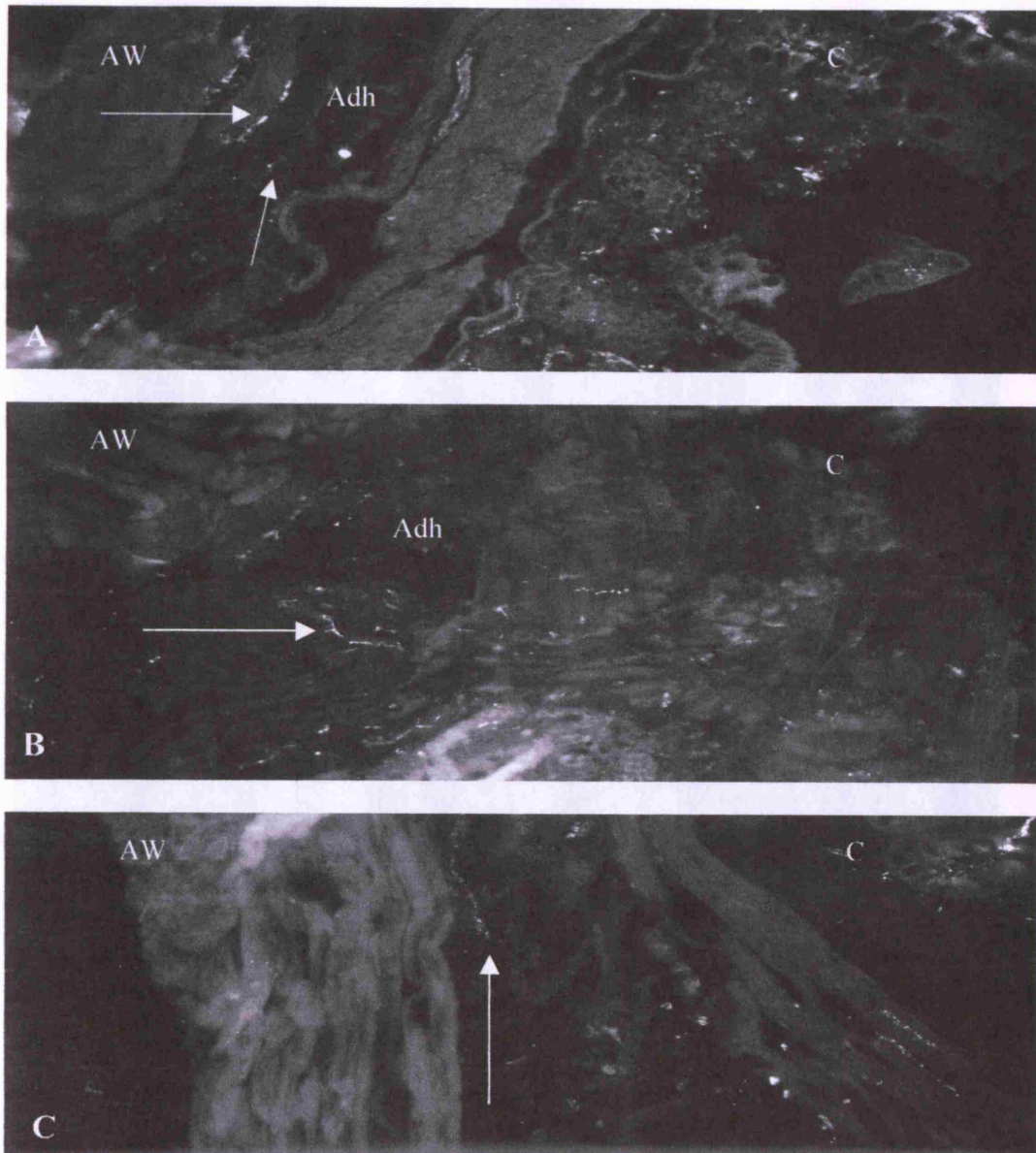


**Figure 3.5.** An adhesion observed between two loops of bowel, at 3 weeks post-surgery. The myenteric nervous plexus of each loop exhibited multiple ganglia and branching calcitonin gene related peptide immunoreactive (CGRP-IR) nerve fibres displaying varicosities (arrow). Multiple nerve fibres arising from one loop of bowel appeared to join other nerve branches from the corresponding nervous plexus of the opposing loop of bowel (x 1470). C: caecum

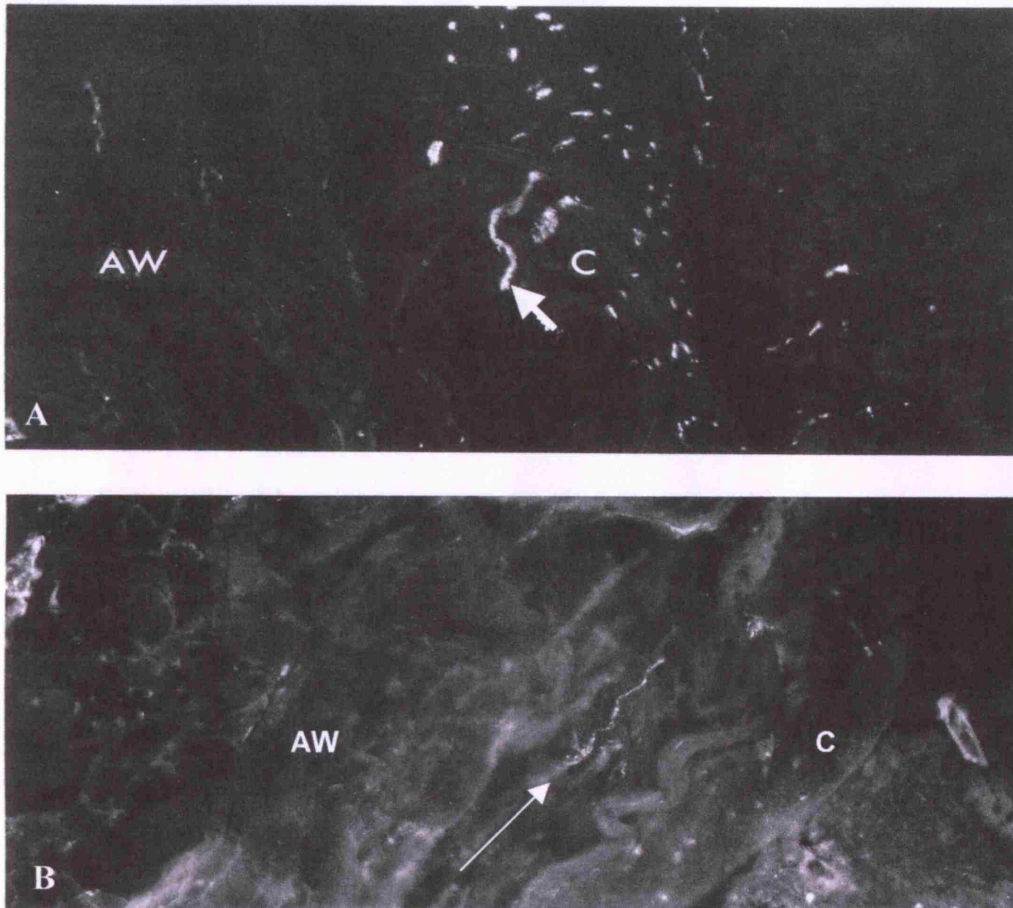


**Figure 3.6.** Substance-P immuno-reactive (SP-IR) nerve fibres in adhesions. (A) Multiple nerve fibres (arrows) were found within the adhesion between the abdominal wall and the caecum at 3 weeks post-surgery. (B) Nerve fibres (long arrow) within the adhesion originating from the caecum, which showed a SP-IR ganglion in the muscle layer (short arrow) at 4 weeks post-surgery. (C) At 8 weeks post-operatively, a long nerve fibre (arrow) was found within the adhesion, parallel to the surface of the abdominal wall (x1470). (C: caecum; AW: abdominal wall; Adh: adhesion).





**Figure 3.7.** Tyrosine hydroxylase immunoreactive (TH-IR) nerve fibres demonstrated in adhesions. (A) Two weeks post-surgery, TH-IR nerve fibres were found adjacent to the abdominal wall (arrows). (B) Multiple nerve fibres were observed in the centre of adhesion (arrow). (C) At 8 weeks post-surgery, a long tortuous nerve fibre with varicosities (arrow) was present in the adhesion tissue between the caecum and the abdominal wall (A-C magnification x1470). C: caecum; AW: abdominal wall; Adh: adhesion.



**Figure 3.8:** Vasoactive intestinal peptide immunoreactive (VIP-IR) nerve fibres were present in post-operative peritoneal adhesion. (A) Three-weeks post-surgery, a nerve fibre was found arising from the caecal wall nervous plexus traversing the adhesion tissue (arrow) (x1250). (B) Eight weeks post-surgery, a nerve fibre with varicosities (arrow) was present in the centre of an adhesion between the caecum and the abdominal wall (x1470). C: caecum; AW: abdominal wall.

### 3.3.4 Vasoactive intestinal peptide (VIP) & Tyrosine hydroxylase (TH)

The distribution of both VIP-IR and TH-IR axons in the control and peritoneal adhesion tissues was similar to those observed in synaptophysin (SYN-IR), calcitonin gene related peptide (CGRP-IR) and substance P (SP-IR) immunoreactive axons (Figure 3.7 and 3.8).

### 3.3.5 Acetylcholinesterase Histochemistry

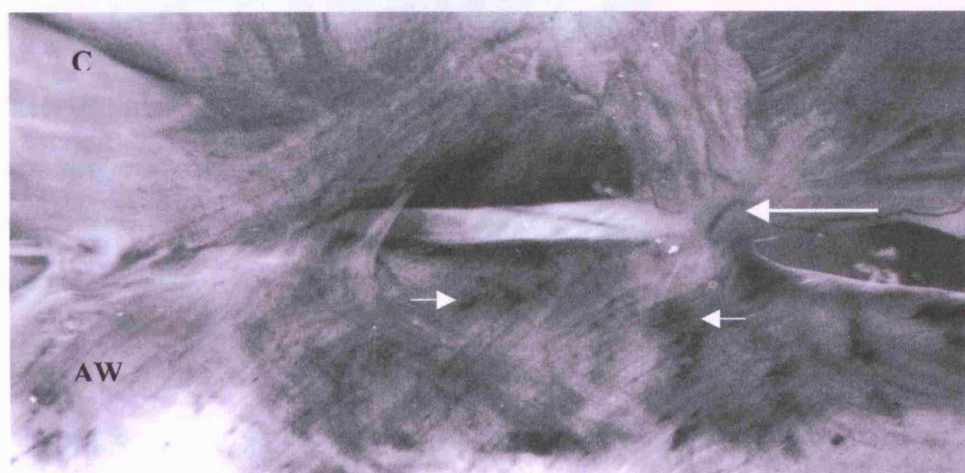
In control tissue acetylcholinesterase histochemistry demonstrated strong staining of neuromuscular junctions (motor end plates) in the abdominal wall and



multiple branching nerve fibres in the caecal wall. However nerve fibres were only detected in adhesion tissue at week 3 post-surgery. With the aid of the dissecting microscope a few thin parallel nerve fibres appeared in the adhesion tissue but their origin could not be determined by this technique. By 4 weeks post-surgery nerve bundles with multiple branching points were found to traverse the entire adhesion (Figure 3.9) and similar findings were observed at 8 weeks.

### 3.3.6 Transmission Electron Microscopy

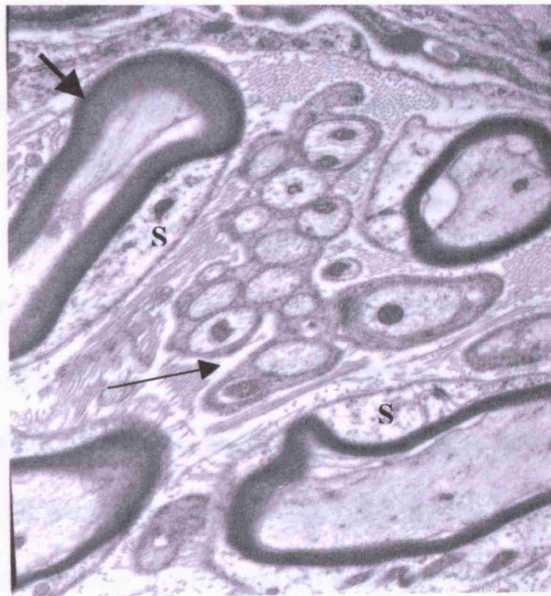
Four weeks after surgery a nerve bundle was observed in the centre of adhesion tissue approximately equidistant from both the caecum and body wall in semi-thin sections stained with toluidine blue (Chapter 2: Figure 2.11). Serial sectioning demonstrated that it originated from a somatic nerve in the abdominal wall. Multiple blood vessels were found close to the nerve bundle.



**Figure 3.9:** Nerve fibres in peritoneal adhesion at 4 weeks post-surgery, shown by acetylcholinesterase histochemistry. A nerve bundle (long arrow) was observed traversing the adhesion with multiple branches in both the caecum (C) and the abdominal wall (AW). Intensely stained sites on the abdominal wall represent neuromuscular junctions (short arrows) (x56).

By eight weeks, further nerve bundles were found within the adhesion. Ultrastructural analysis of one of the nerve bundles (Fig. 3.10) demonstrated multiple

myelinated and non-myelinated nerve fibres with accompanying Schwann cells encircled by the surrounding perineurium. The non-myelinated axons appeared to have a smaller diameter than the myelinated axons. In control tissues normal histological findings were seen in caecum and anterior abdominal wall.



**Figure 3.10:** A nerve bundle demonstrated within the adhesion tissue by transmission electron microscopy. Myelinated (thick arrow) and unmyelinated nerve fibres (thin arrow) with accompanying Schwann cells (S) were found within the adhesion. Abundant collagen fibres were present adjacent to nerve fibres (TEM, x 6000).

### 3.4 DISCUSSION

This study provides the first direct evidence that nerve fibres grow into peritoneal adhesions following a reproducible serosal injury model in an animal. Nerve fibres immunoreactive for synaptophysin, calcitonin gene related peptide (CGRP), substance P (SP), vasoactive intestinal peptide (VIP) and tyrosine hydroxylase (TH) were observed within adhesions from two weeks post-surgery (see Appendix A3) and appeared to originate from the caecal wall. The presence of CGRP a neuropeptide that is abundant in sensory nerve fibres (Gibbins, 1987) suggests that some of the nerve fibres within the newly formed adhesions are sensory. In addition substance P-immunoreactive axons were found extending from the caecum two weeks after surgery implying that a proportion of these sensory fibres are capable of the transmission of pain stimuli. Acetylcholinesterase histochemistry clearly demonstrated that some nerve fibres spanned the length of the adhesion. Furthermore ultra-structural analysis showed adhesions contained both myelinated and non-myelinated axons, the latter appeared thinner in diameter suggesting that they may be C or A- $\delta$  fibres, which are known to be pain-conducting fibres (Tortora and Grabrowski, 1996).

The origin of the substance P-immunoreactive fibres within the adhesions is not clear as there are several possible sources. Sensory fibres which arise from the caecal wall are connected with the enteric nerves particularly those of the myenteric plexus which contains afferent neurons including some that express substance P (Furness et al., 1995; Kunze et al., 1995). They are also connected to the prevertebral ganglia of the sympathetic nervous system, the spinal cord and some fibres in the parasympathetic nervous system (the vagi for instance) that tracks back to the brain stem. These sensory nerves elicit local reflexes within the gut itself and also reflexes that are relayed back to the gut from either the prevertebral ganglia or the central nervous system. The caecal wall also receives sensory fibres from dorsal root ganglia neurons which are also

substance P- and /or CGRP- immunoreactive. The source of the sensory nerve endings extending from the parietal peritoneum probably originate from somatic nerves and have cell bodies in the sensory dorsal root ganglia transmitting impulses from the overlying skin and muscle and hence via the dorsal roots to the spinal cord. The axons of motor neurones emerge in the ventral root and end in motor end-plates of voluntary muscles. These nerves contain afferent fibres, including some expressing CGRP and substance P.

It has been suggested that the surgical technique and the degree of injury have a major effect on the rate of nerve regrowth (Holland et al., 1995). In the present study, the parietal peritoneum overlying the abdominal wall was extensively damaged and this extended to the muscle layer. The caecum however received a relatively mild trauma as gentle scraping damaged only surface mesothelium and the adjacent submesothelial layer. The greater intensity of trauma applied to the parietal peritoneum may explain the delayed appearance of nerve fibres originating from the body wall. Nerve growth was also seen in the submesothelial layer of both the visceral and the parietal peritoneum adjacent to the main trauma sites. These areas displayed increased extracellular matrix deposition with extensive blood vessel formation compared with the peritoneum of control tissue which was devoid of nerves. This finding suggests that damage to the serosal surface, however mild, can stimulate axonal growth into and through the submesothelial layer.

The rate of nerve growth demonstrated in this study was similar to that reported in a previous study examining extrinsic adrenergic nerve regrowth in a rat model of small bowel transplantation (Kiyochi et al., 1993). The distribution of afferent nerves in a rat model of bladder denervation (Gabella and Davis, 1998) was comparable with our results. Cholinergic characteristics are acquired relatively early and the appropriate phenotypic expression may be promoted by cholinergic differentiation factor and

cholinergic (vagal) fibres were found to regenerate between 6-24 months after cardiac transplants in humans and after complete vagal nerve transection in dogs (Valentin et al., 1987). In this study the sympathetic adrenergic fibres arose from the caecum and passed across the adhesion tissue towards the body wall. Hill et al. (1985) similarly observed re-innervation of both arterial smooth muscle cells and enteric intrinsic neurons to be predominantly by sympathetic fibers regenerating from interrupted paravascular nerve bundles. In the gut the early regenerating sympathetic fibers grow along submucosal blood vessels only as a pathway into the gut and then ramify extensively over the first ganglia encountered. The sympathetic fibres re-innervating both the arteries and the intramural plexuses regenerate from the interrupted axons of the paravascular nerve trunks.

The factors regulating nerve growth were not examined in this study, but other reports suggest that the local environment is a major factor which controls the appropriate differentiation of the prospective autonomic ganglion neurons. It is likely that the same trophic factors released following injury (nerve growth factor, brain-derived neurotrophic factors, and neurotrophins) and extracellular matrix components involved in contact guidance (laminin, fibronectin, and tenascin) which have been shown to regulate nerve growth in other systems (Fu & Gordon, 1997) play a role in the directional migration of nerves into post-operative adhesions. Other growth factors include the fibroblast growth factors (FGFs) and ciliary neurotrophic factor (CNTF). Furthermore, angiogenesis is thought to contribute to the innervation process during normal tissue repair. In healing skin grafts nerve fibres were shown to migrate along invading blood vessels (Manek et al., 1993) and perivascular patterns of innervation have been described following bowel transplant studies (Sugitani et al., 1998). In the present study blood vessels were fully developed in the adhesions by one week and may have guided the subsequent migration of axonal sprouts through the adhesion tissue.

Several studies have dealt with the relationship between the presence of foreign bodies such as those used in operative procedures (glove powder, gauze, and sutures), and adhesion formation (McEntee et al., 1987; Luijendijk et al., 1996). The inflammatory response whether elicited by these materials or by underlying inflammatory bowel disease such as ulcerative colitis or Crohn's disease may produce a multitude of growth factors which stimulate the vascularization and innervation of developing adhesions. The presence of non-absorbable sutures with an associated inflammatory response may have induced the growth of nerves into the adhesions in this study.

There is little doubt about the role adhesions play in intestinal obstruction (Ellis, 1998) and infertility in women (Diamond et al., 1987; Sekiba et al., 1992), but there is considerable controversy about their importance in the aetiology of recurrent abdominal pain (Rapkin, 1986; Alexander-Williams, 1987). Numerous studies have implicated adhesions in the differential diagnosis of chronic abdominal pain (Kresch et al., 1984; Trimpos et al., 1990; Stout et al., 1991) and many patients are relieved of pain after adhesiolysis (Steege and Stout, 1991; Peters et al., 1992). The concept of pain perception has grown more complicated with time and integrates the influence of personality, organic changes, sensory thresholds and cognitions. The modern ideas about pain and its causes, avoid a clear demarcation between psychological and physical causes for pain but rather recognizes their intricate relationship (Steege and Stout, 1991).

Although the present study demonstrated the presence of sensory nerve fibres, which are likely to include pain-conducting fibres it is still not known whether chronic abdominal pain is due to stimulation of pre-existing pain fibres in regions adjacent to adhesions (Lundberg et al., 1973; Keltz et al., 1995), or to stimulation of sensory nerve fibres within the adhesions themselves. Almeida et al. (1997) conducted a prospective

study of 50 women with chronic pelvic pain who underwent microlaparoscopy under local anaesthesia with sedation. Pelvic adhesions were found in 31 (62%) of these patients. The same group subsequently performed conscious pain mapping by systematically probing the pelvic structures and obtaining intra-operative patient feedback for the presence or absence of pain (Almeida and Val-Gallas, 1997). Twenty-one (80%) of the patients with adhesions felt tenderness when these structures were touched suggesting that adhesions were capable of inducing pain sensations probably via sensory fibres.

In conclusion, this study has shown the presence of sensory nerve fibres within the post-operative abdominal adhesions in a murine model, from as early as 2 weeks after injury. Adhesions may be indirectly involved in the aetiology of chronic abdominal pain by restricting the mobility of adjacent organs and causing an inordinate degree of stretching of the smooth muscle and thus presumably some pain. However the presence of thin unmyelinated nerve fibres within adhesions, may enhance the sensitivity of the area and thus possibly in the conductance of pain sensation in the abdomen.

Nerve fibres have been demonstrated in human pelvic (Kligman et al., 1993; Tulandi et al., 1998) and abdominal adhesions (Herrick et al., 2000) but their morphology and pattern of growth had not been examined. Adhesions formed in our post-surgical murine model were similar to human adhesions, irrespective of any underlying pathology (Herrick et al., 2000). Histologically both are formed of similar cellular elements, extracellular matrix with abundance of collagen deposition, and blood vessels. Therefore, this highlights the strong possibility that a similar mechanism of nerve growth occurs in humans irrespective of whether adhesions are due to infection or surgery. The following Chapter will examine human peritoneal adhesion tissues to assess the presence of sensory nerves and to characterize their type and orientation.

## **CHAPTER FOUR**

### **PRESENCE AND DISTRIBUTION OF NERVES IN HUMAN**

#### **PERITONEAL ADHESIONS**



## 4.1 INTRODUCTION

Post-operative peritoneal adhesion formation occurs in 93-100% of patients undergoing laparotomy (Menzies and Ellis, 1990) leading to complications such as adhesive intestinal obstruction and infertility in women (Bevan, 1984; DeCherney et al., 1984). However, the clinical significance and relationship of chronic abdominal/pelvic pain with peritoneal adhesions is unclear (Rapkins, 1986; Alexander-Williams, 1987). Chronic abdominal/pelvic pain is a significant clinical problem and accounts for more than 25% of all gynaecological visits and more than 40% of all laparoscopic procedures in the USA (Howard, 1994). Numerous studies have implicated adhesions in the differential diagnosis of chronic abdominal pain (Kresch et al., 1984; Trimbos et al., 1990; Stout et al., 1991) and many patients are relieved of pain after lysis of adhesions (Goldstein et al., 1980; Sutton & Macdonald, 1990; Steege and Stout, 1991; Peters et al., 1992). Conscious pain mapping studies using micro-laparoscopic techniques found that 80% of patients with significant pelvic adhesions complained of tenderness when these structures were probed (Almeida et al., 1997), suggesting that these structures can generate pain stimuli.

Kligman et al. (1994), found nerve fibres in 58% of pelvic adhesion specimens by immunolocalisation of a Schwann cell marker (S-100). Tulandi and colleagues (1998) demonstrated nerve fibres in 78% of pelvic adhesions studied using a neurofilament marker. Herrick et al. (2000) found nerve fibres in approximately 60% of human peritoneal specimens examined histologically using Linder's silver nerve stain. None of these studies identified the nerve fibres. In previous chapter we showed the presence of nerve fibres in post-operative peritoneal adhesions in all the specimens assessed in the animal model.

The aim of the present study was to characterise the type and distribution of nerve fibres formed in human peritoneal adhesions and to relate the findings to the

location, size and age of the adhesion and clinical parameters such as reports of chronic abdominal/pelvic pain. Peritoneal adhesions were collected and processed for histological, immunocytochemical and ultrastructural analysis. As in Chapter 3 for murine adhesions, nerve fibres were characterised using specific antibodies against a protein in synaptic vesicles (synaptophysin) as well as specific neuropeptides such as calcitonin gene-related peptide (CGRP) expressed by sensory nerves and Substance P present in pain conducting fibres. Fibres expressing vasoactive intestinal peptide (VIP), a marker for the enteric nervous system, and tyrosine hydroxylase (TH), an intermediary enzyme in catecholamine synthesis, for the autonomic nervous system were also immunolocalised. The presence of myelinated and non-myelinated axons was determined ultra-structurally by transmission electron microscopy. Nerve fibre distribution was analysed by acetylcholinesterase histochemistry in whole-mount specimens, and also compared with that of blood vessels using dual immunolocalisation, where sections were initially immuno-stained for neuronal markers, and then were immuno-stained with primary antisera against von Willebrand factor. Von Willebrand Factor is synthesized by endothelial cells, causes adhesion of platelets to injured vessel walls, and functions as a carrier and a stabilizer for coagulation of Factor VIII.

## 4.2 MATERIALS AND METHODS

All chemicals were obtained from Sigma (Poole, Dorset, UK) or BDH (Lutterworth, UK) unless otherwise stated.

### 4.2.1 Patients' clinical information

Twenty-five patients with abdominal or pelvic adhesions undergoing laparotomy for various reasons were included in this study. Ethical committee approval for the study was obtained and all patients were consented prior to their participation in this study. The group comprised 10 males and 15 females patients, aged 18 to 81 years. The indications for surgery included: colon cancer (five); ulcerative colitis (three); adhesive intestinal obstruction (two); fibroids (five); ovarian cyst (two); cancer cervix/ovary (three); persistent pelvic pain (one); others such as appendicectomy, cholecystectomy and hernia (four). Twenty patients had a history of abdominal/pelvic pain of more than three months duration. Seven of the 25 patients had no previous history of abdominal/pelvic surgery. Patients' details and surgical history are presented in Tables 4.1 and 4.2.

**Table 4.1:** Summary of patients' surgical data

Number of patients	Indication for surgery	Patients with previous surgery	History of pain Pre-operatively
5	Cancer colon	3/5	4
3	Ulcerative colitis	2/3	2
5	Fibroids	4/5	3
2	Ovarian cyst		2
3	Cancer cervix/ovary	2/3	3
2	Adhesive intestinal obstruction	2/2	2
1	Persistent pelvic pain	1/1	1
4	Others: cholecystectomy, appendicectomy, ventral hernia	3/4	3
<b>TOTAL</b>			
25		18	20

**Table 4.2: Details of patients' clinical data**

No.	Sex	Age	Diagnosis	Past Medical history	Drug History	Operation	Site of Adhesion	No. of Adhesions	Hx. Of Abd/pelv. Pain
1	F	81	Rt. ovarian cyst	Schizo-Phrenia	For Schizophrenia	ovarian cystectomy	Rt. ovary-uterus	1	No
2	F	37	Pelvic pain	Nil	N/A	Laparoscopy/Adhesiolysis	L. ovary-lat. Pelvic wall	1	Yes
3	F	75	Fibroid+R. ovarian cyst	Appendectomy 1965; colpo-suspension 1975	Tildiem - Persantin Steriods- slow K	Hysterectomy	Uterus-R. ovary; Uterus-colon	2	Yes
4	M	47	closure of ileostomy (ca. colon)	colectomy & defunc. Colostomy 1997 Re-laparo + ileostomy 1998	Nil	Closure of ileotomy	S/B - S/B	6	Yes
5	F	73	Ovarian cyst (left)	Varicose veins 1970	NSAIDs	Ovarian cystectomy	Lt. ovary-fallopian tube	1	Yes
6	F	65	Ca. Sigmoid colon	Appendectomy 1948; DM, COAD, Hypertension	Steriod, HRT, Aspirin, Metformin	Lt. Hemicolectomy	Colon-S/B	1	Yes
7	F	74	Fibroid	N/A	N/A	Hysterectomy + BSO	Uterus-bladder; Colon-fallopian tube	2	No
8	F	66	Ca. colon	Caesarian Sections x2 Cholecystomy 1986; Ventral Hernia 1997	Nil	Extended Right Hemicolectomy	Colon-colon	3	Yes
9	F	49	Pelvic mass (fibroid)	Caesarian Sections x6; Ovarian abscess 1992	Nil	Hysterctomy + BSO	Uterus-Abd. Wall; Uterus-ovary; Colon-abd. Wall; Colon-colon	6	Yes
10	M	74	Chole-cystitis	Parkinson's disease; Rec. biliary colics	Sinemet Entacapone	Open cholecystectomy	Gall bladder-duodenum	1	Yes
11	F	59	Vag. bleeding / Endometrial hyper-plasia	Nil	Nil	Hysterectomy + BSO	Tube-ovary	1	No
12	F	36	Incisional hernia	Asthma Laparotomy + Appendectomy 1978; C.S. 1993-1998 Incis. hernia repair 1998	Salbutamol Steriods	Repair of Incisional hernia	Omentum-abd. Wall; omentum-colon	5	Yes
13	F	57	Cancer ovary	Laparotomy (fibroid) 1984 TAH: 1989	Nil	Laparotomy + Radical Oophorectomy	Omentum - Abd. wall	1	Yes
14	F	47	Cacer cervix in situ	Laparoscopy x2: 1983 Laparotomy: 1985 (for endometriosis)	Nil	TAH + BSO	Tube - uterus	1	Yes
15	F	69	Fibroids	laparoscopy	Dexa-	TAH + BSO	Uterus - colon	1	No

				x2: 1998-1999	amphetamine co-amilofruse				
16	F	53	Fibroids + ovarian mass	D & C: 1998 Melanoma (R) arm 1983. Hypertension	Atenolol	TAH + BSO	Ovary - lateral pelvic wall	1	Yes
17	F	50	Fibroid	D & C	Arnica	Subtotal Hysterectomy + BSO	Uterus - bowel	1	Yes
18	F	69	Cancer colon	Gastrectomy: 1979	Nil	Anterior resection	Liver - abd. wall S/B - S/B	2	Yes
19	F	48	Anal sphincter disorder	Loop colostomy: 1997	Nil	End colostomy	colon- abdominal wall	1	No
20	M	83	Adhesive Subacute Intestinal Obstruct.	Colectomy: 1986 (ca colon)	NSAIDs Adalat Prozac	Laparotomy adhesiolysis +	S/B - S/B	5	Yes
21	F	46	Cancer colon	Ruptured ectopic preg.	N/A	Extended hemicolectomy R.	Colon - abd. Wall	2	No
22	M	39	Ulcerative colitis (U.C.)	Pouch formation: 1997	N/A	Pouch formation	S/B - S/B	2	No
23	M	18	U. C.	Colectomy: 1996	N/A	Reversal colostomy adhesiolysis of +	colon - S/B	1	Yes
24	M	38	U. C.	Colectomy: 1997	N/A	Rectal resection + pouch formation	Colon - abd. wall colon - S/B	2	Yes
25	M	57	Adhesive Intestinal Obstruct.	Appendec- tomy 1980	Nil	Laparotomy adhesiolysis +	Colon - abd. wall S/B - S/B	2	Yes

U.C.: Ulcerative colitis; DM: Diabetes mellitus; Ca.: Cancer; S/B: Small bowel; BSO: Bilateral salpingo-  
ophorectomy; D&C: Dilatation & curettage; Rt.: Right; Lt.: Left; TAH: Total abdominal hysterectomy;  
N/A: Not applicable; Hx. of Abd/Pelv: History of Abdominal/Pelvic.

#### 4.4.2 Tissue processing

All peritoneal adhesion specimens (n = 52) were collected intra-operatively. Site, size and number of adhesions were noted. Common sites of adhesion formation were between loops of small bowel, small bowel and colon, omentum and small bowel, uterus and colon, small bowel and anterior abdominal wall. Macroscopically, adhesions ranged in length from 0.5 to 5 cm. Adhesions were excised from between serosal surfaces and the central portion collected and briefly washed in PBS pH 7.4, and then immediately placed in appropriate fixative medium according to the subsequent tissue processing. All adhesions were assessed histologically and immunocytochemically, but

only 10 specimens were prepared for acetylcholinesterase histochemistry (n = 5) and transmission electron microscopy (n = 5).

#### **4.2.3 Histological studies**

For histological studies, the tissue was immediately placed in 4% paraformaldehyde in PBS, pH 7.4. The tissue was fixed overnight at 4°C and paraffin wax embedded. Wax blocks were sectioned at 5µm using a microtome (Pabish, Germany) and stained with Haematoxylin & Eosin and Masson's Trichrome using standard protocols (see Appendix 1 and 2).

#### **4.2.4 Immunocytochemical studies**

Tissue was fixed with 4% paraformaldehyde in PBS, pH 7.4 for one hour at room temperature, then washed in PBS for 30 minutes, transferred to 7% sucrose with 0.1% sodium azide ( $\text{NaN}_3$ ) in PBS, pH 7.4 and stored overnight at 4°C. Specimens were embedded in Optimal cutting compound (OCT, Miles Inc. Elkhart, USA) and snap frozen over liquid nitrogen. Frozen sections (10µm) were cut with a cryostat and stored at -20°C until used. Sections were washed with PBS and then incubated overnight at room temperature with well characterized primary antibodies (1:1000 optimal dilution) raised against synaptophysin (Rabbit polyclonal against human synaptophysin; Dako, Glostrup, Denmark), substance P (Rabbit polyclonal against human substance P; Chemicon International, Harrow, UK), CGRP (Rabbit polyclonal against rat calcitonin gene-related peptide; Affiniti, Exeter, UK), tyrosine hydroxylase (TH) (Rabbit anti-rat TH; Chemicon International, Harrow, UK), and vasoactive intestinal peptide (VIP) (Rabbit anti-rat VIP; Chemicon International, Harrow, UK). After washing three times with PBS sections were incubated for one hour at room temperature with biotinylated donkey anti-rabbit IgG (Amersham, Bucks, UK) diluted at 1:250. Following a PBS wash three times, the sections were incubated for one hour with streptavidin-fluorescein

reagent diluted at 1:100 (Amersham, Bucks, UK) mounted with Citifluor (Canterbury, Kent, UK) and coverslipped. Control sections were incubated with either normal blocking serum or rabbit IgG instead of primary antisera. All sections were examined with a microscope equipped with fluorescent filters (Axiophot microscope, Zeiss, Germany). For double immunolocalisation sections were initially immuno-stained, as described for neuronal markers and then the same sections were immuno-stained with primary antisera against von Willebrand factor. Anti-sera to von Willebrand Factor (vWF) (sheep anti-human vWF, Chemicon International, Harrow, UK) was diluted 1:1000 and incubated on sections for one hour at room temperature followed by incubation for one hour with rhodamine-conjugated secondary antisera (anti-sheep IgG-rhodamine, Chemicon International, Harrow, UK) diluted 1:500 at room temperature in a humidified chamber. Sections were visualised using a microscope equipped with fluorescent filters (Axiophot microscope, Zeiss, Germany).

#### **4.2.5 Acetylcholinesterase histochemistry**

Acetylcholinesterase histochemistry was performed according to the method of Baker et al., (1986). Briefly, excised adhesion specimens were pinned onto a slab of slygard (Dow-Corning, Weisbaden, Germany) as a wholemount preparation and fixed for one hour in 10% buffered formalin, pH 7.2 at 4°C. Specimens were then washed in 100 ml PBS, pH 7.2, containing 1280 units of Hyaluronidase (Sigma, Poole, UK) and 10<sup>-4</sup>M tetraisopropylpyrophosphoramidate (iso-OMPA; Sigma, Poole, UK) and left overnight at 4°C. Tissue was then incubated with acetylcholine solution (5mg acetylcholine iodide, 6.5ml 0.1M acetate buffer containing 1.5% Triton X-100, 0.5ml 0.15M sodium citrate, 1ml 30mM copper sulphate, 10ml 1mM iso-OMPA and 1ml 5mM potassium ferric cyanide) for 24 hours with at least four solution changes. The specimens were regularly viewed under a stereo-microscope (Zeiss, Germany) and when staining was sufficiently intense the reaction was stopped by immersing the

specimen for one hour in 10% buffered formalin and followed by a PBS wash. Tissue was mounted in 70% glycerol between glass slides, viewed using a stereo-dissecting microscope (Zeiss, Germany) under epi-illumination, and photographed using Ilford HP5 400 ASA black and white film.

#### **4.2.6 Transmission electron microscopical (TEM) studies**

Adhesion specimens were fixed with a mixture of 1% paraformaldehyde and 5% glutaraldehyde in 100 mM Na cacodylate buffer, pH 7.4 overnight at room temperature. Tissue was then washed in fresh 100mM Na cacodylate buffer and post-fixed in 1% osmium tetroxide in 100mM Na cacodylate buffer pH 7.4 for one hour. Specimens were re-washed in cacodylate buffer, stained in an aqueous saturated solution of uranyl acetate for one hour, dehydrated and embedded in araldite epoxy resin as described in Chapter 2, section 2.3.3. Semi-thin sections (0.5-1  $\mu\text{m}$ ) were prepared using a microtome (Reichert, Austria), stained with 1% toluidine blue (Sigma, Poole, UK) and mounted with a drop of Araldite resin. Sections were examined under a Zeiss Axiophot light microscope equipped with phase contrast optics. Ultra-thin sections (70-100nm) of particular areas of interest were collected on copper grids, stained with aqueous saturated solution of 3% uranyl acetate in 50% alcohol and lead citrate (Sigma, Poole, UK), viewed and photographed with a Philips 400 transmission electron microscope (Eindhoven, Holland) using Kodak electron microscope 4489 film.



## **4.3 RESULTS**

### **4.3.1 Histological assessment of human adhesion specimens**

Fifty-two peritoneal adhesion specimens were collected from 25 patients (range of 1-6 specimens/patient); 9 were pelvic adhesions (uterus-fallopian tubes-ovaries-lateral pelvic wall), 37 abdominal adhesions (colon-small bowel-omentum-anterior abdominal wall-liver-gall bladder), and 6 combined pelvic and abdominal adhesions (uterus-colon; fallopian tube-colon; uterus-abdominal wall). Adhesion size varied, although it was only the central portion of adhesion tissue that was of interest. All specimens were collected in patients that had between 1-3 adhesions, while patients with 4-6 adhesions, only 3 specimens were collected. Forty-two peritoneal adhesion specimens were therefore studied in total (42/52). Age and sex of patients did not seem to influence the existence, size, number or location of adhesions. Past medical history contributed to the existence of adhesions in 18/25 patients due to a previous pathology or surgery, whereas 7 patients did not have any previous pathology or surgery. Estimated age of the adhesions based on previous pathology or surgery ranged from 9 months to 51 years. The site of adhesion depended on the primary pathology in most cases (23/25), for example adhesions appeared both filmy and fibrous with evidence of vascularity. Tight adhesions, where just a tiny or broad band of tissue existed between 2 closely opposed organs could not be collected, as that would cause damage to the surface of the structures involved. Therefore, adhesions had to be around or more than 1 cm to be collected safely.

Histological assessment demonstrated that in general human peritoneal adhesions showed both areas of loose and dense bundles of collagen fibres combined with adipose tissue as described for day 7 mouse adhesions in Chapter 2. Pelvic adhesions appeared more fibrous in comparison to abdominal adhesions, but no quantitative studies were performed to verify this. Some peritoneal adhesions collected from the upper abdominal

cavity showed clear zones of demarcation between the adipose and fibrous tissue where the omentum was involved (6/52). All adhesions were well vascularized (Figure 4.1 A) and in some specimens, nerve fibres were clearly identified following H&E and Masson's trichrome staining (Figure 4.1B).

#### **4.3.2 Demonstration of neuronal markers in human adhesions by immunohistochemistry**

Nerve fibres were clearly identified in all abdominal and pelvic adhesion specimens examined immunocytochemically for neuronal markers. They appeared more abundant in abdominal adhesions compared with the pelvic adhesions, although quantitative studies were not performed. Nerve fibres in serial sections were immunoreactive for all the neuronal markers assessed: calcitonin gene-related peptide (CGRP) (Figures 4.2 and 4.3), substance P (SP) (Figure 4.4), synaptophysin (Figure 4.5 A), tyrosine hydroxylase (TH) (Figure 4.5 B) and vasoactive intestinal peptide (VIP), irrespective of location, size. Furthermore, the surgical history of patients or the estimated age of the adhesion did not appear to affect the presence of nerve fibres. Adhesions from the 5 patients without a history of chronic abdominal/pelvic pain also displayed sensory nerve markers (CGRP and SP).

Nerves consisted of either single axons with a distinct beaded or varicose appearance or bundles of fibres lying in parallel. When localised with all neuronal markers, long and short single axons immunoreactive for CGRP were found in the interstitial as well as in the adventitia of blood vessels (Figure 4.3). Most nerve fibres irrespective of nerve marker used co-localized with blood vessel markers as demonstrated by dual immuno-staining for von Willebrand factor, a marker of endothelial cells (Figures 4.2, 4.4 and 4.5). However, it was obvious that others appeared to be independent of BVs. TH-immunoreactive axons were fewer in number than those identified using the other markers, although they were abundant within the

adventitia of blood vessels. The distribution of nerve fibres in all peritoneal adhesion specimens appeared to be similar irrespective of whether they are abdominal or pelvic. Overall, more fibres appeared immunoreactive for CGRP, then Synaptophysin and SP, followed by TH and VIP suggesting an abundance of sensory nerve fibres in adhesions. Negative control sections showed no immunoreactivity.

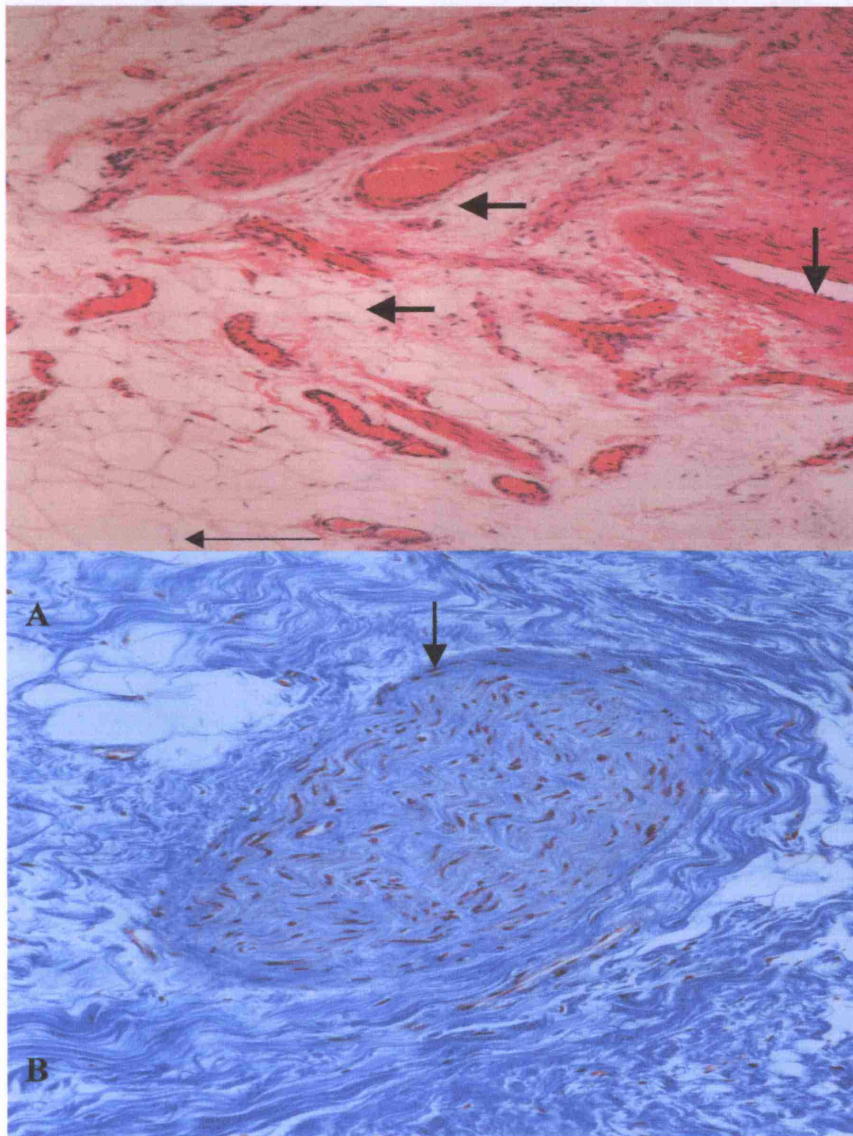
#### **4.3.3 Acetylcholinesterase Histochemistry**

A portion of 5 human peritoneal adhesion specimens from the 52 specimens studied were prepared for wholemount preparation following acetylcholinesterase histochemistry. All adhesions displayed blood vessels that appeared opaque and nerve fibres that appeared dark (Figure 4.6) following the procedure. In general, nerve fibres appeared associated with blood vessels and were in parallel to the longitudinal axis of the adhesion. However, as shown by immunocytochemistry, some nerve fibres with multiple branching points appeared independent of blood vessels and transversed across the adhesion as a neuronal meshwork. All 5 specimens studied appeared similar in appearance.

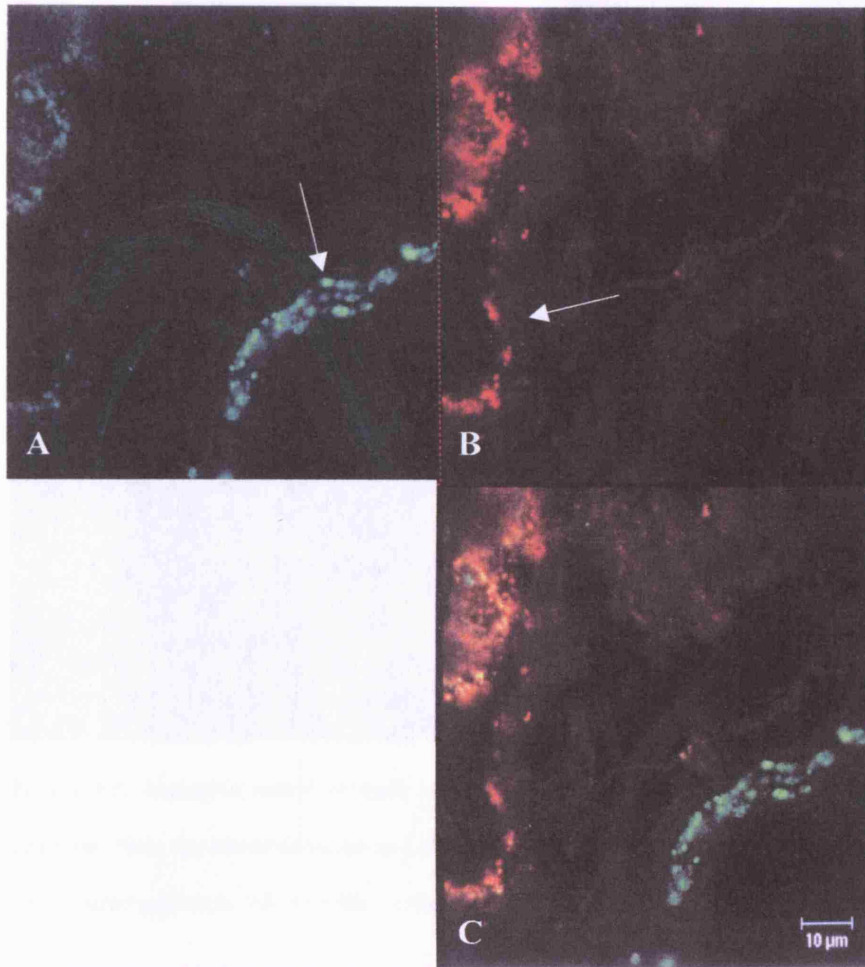
#### **4.3.4 Transmission Electron Microscopy**

Ultrastructural examination of a selection of human adhesion samples from the 52 specimens studied revealed nerve bundles with surrounding perineurium. Nerves were composed of myelinated and non-myelinated fibres with accompanying Schwann cells (Figure 4.7). Non-myelinated fibres appeared thinner than myelinated fibres. In transverse section some nerve fibres with accompanying collagen fibrils were sectioned transversely and others obliquely suggesting that some nerve fibres were not oriented with respect to the longitudinal axis of adhesion. All 5 specimens showed myelinated and non-myelinated fibres although some specimens (3/5) showed more nerve fibres than others and were from patients who had previous abdominal/pelvic surgery and experienced pre-operative abdominal pain. The other 2 specimens were one from a

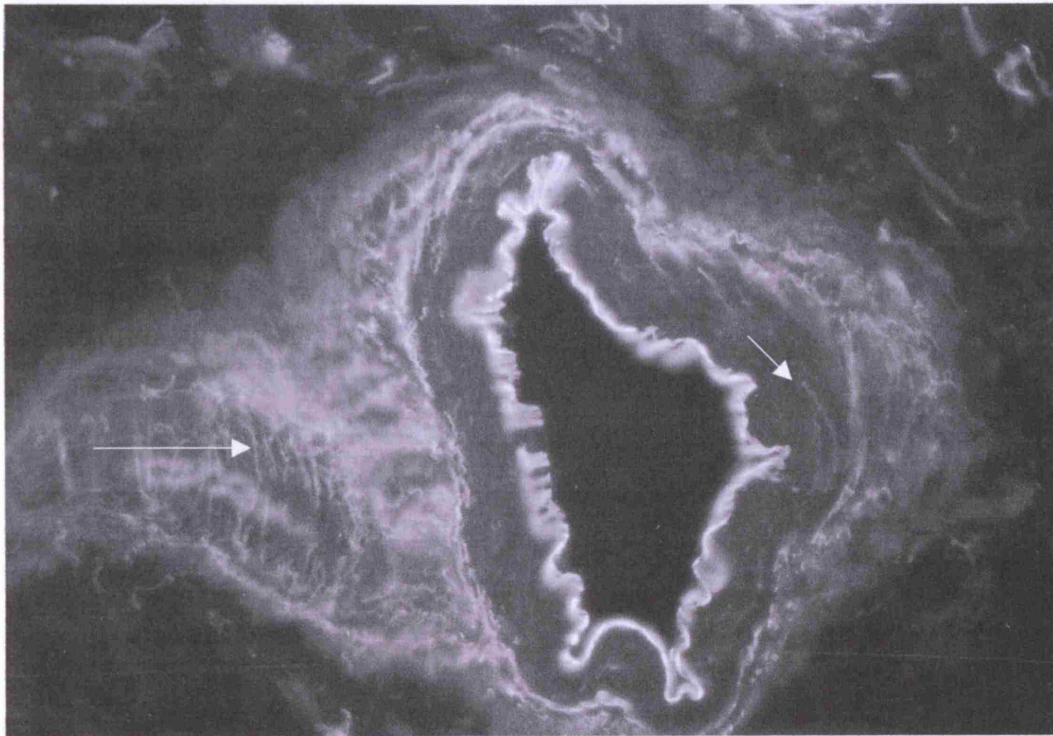
patient who had no associated pre-operative abdominal pain, while the other patient experienced the pain. However, the number of nerve fibres was not quantified.



**Figure 4.1:** (A) Human peritoneal adhesions, showing adipose tissue with the signet ring-like appearance of fat cells (arrow) adjacent to collagenous connective tissue. Adhesions were highly vascularized (thick arrows) (H & E; x430). (B) Human peritoneal adhesion, showing a nerve bundle (arrow) in the middle of the adhesion surrounded by dense collagen bundles and adipose tissue (Masson's trichrome, x430).

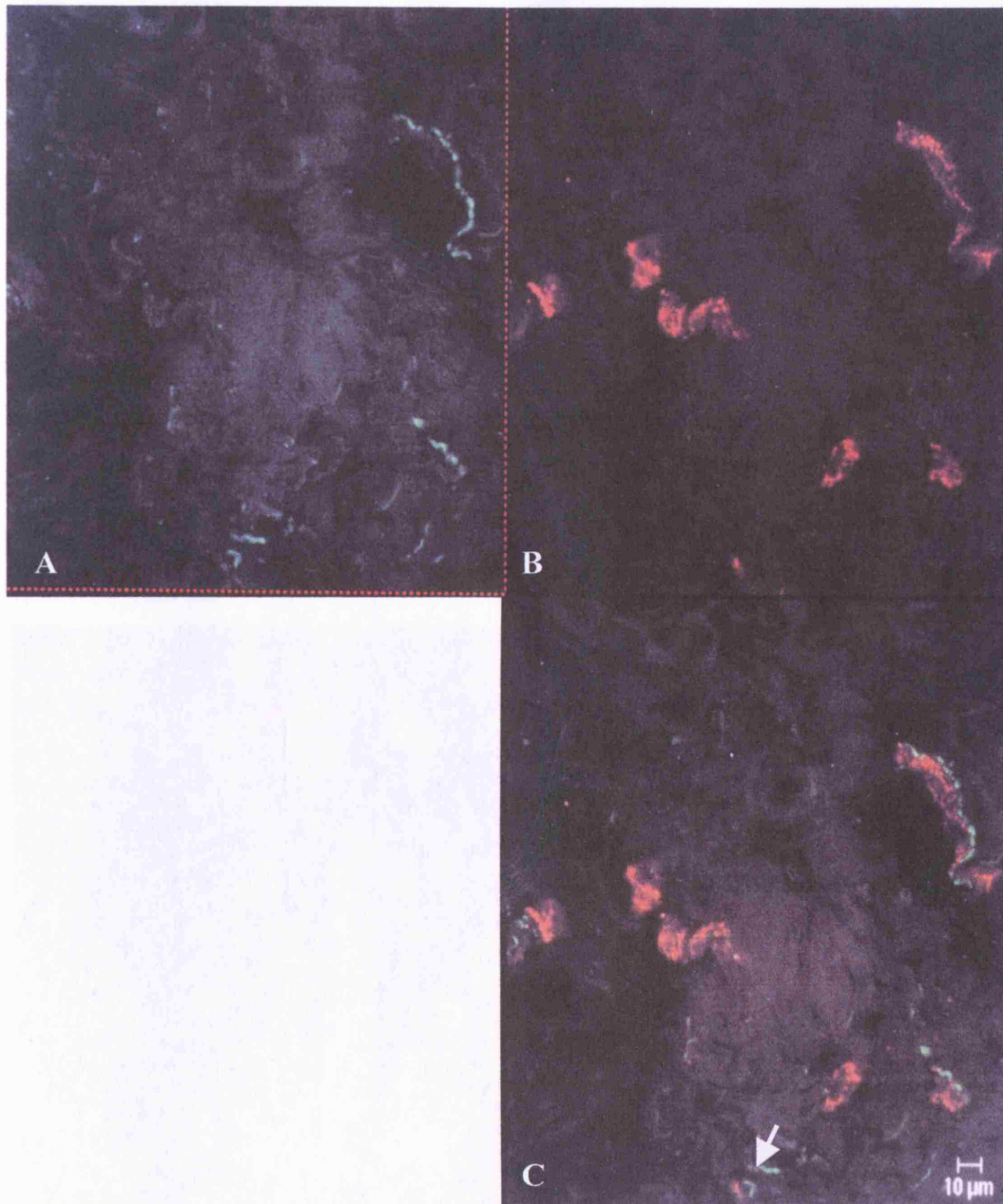


**Figure 4.2:** Calcitonin gene related peptide-immunoreactive nerve fibres in human adhesions. (A) Nerve fibre (green) shows typical varicosities in parallel or beaded appearance (arrow). (B) A blood vessel (red) immunoreactive for von Willebrand factor (arrow). (C) Co-localization of the nerve fibre (green) with blood vessel (red). Bar represents 10  $\mu\text{m}$ .

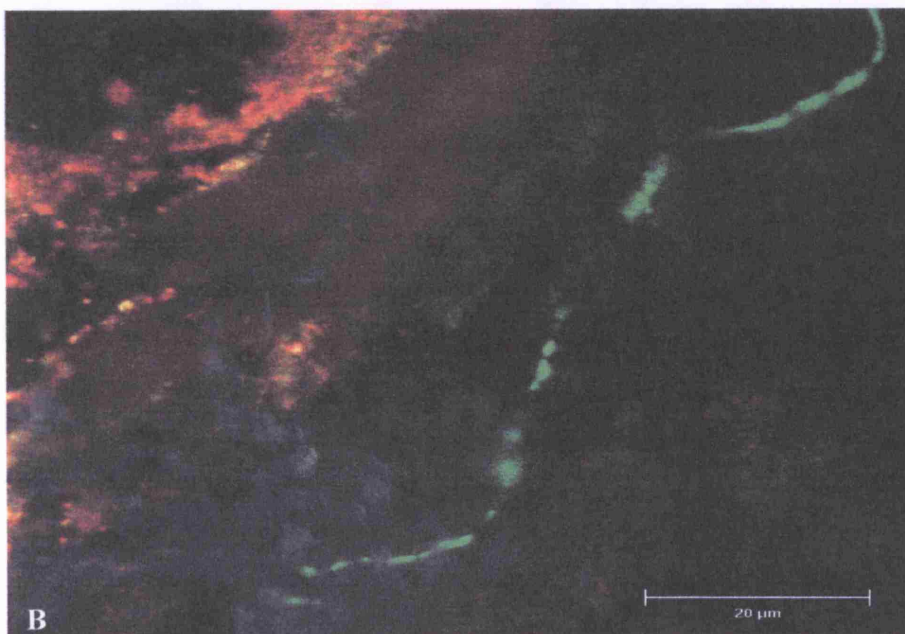
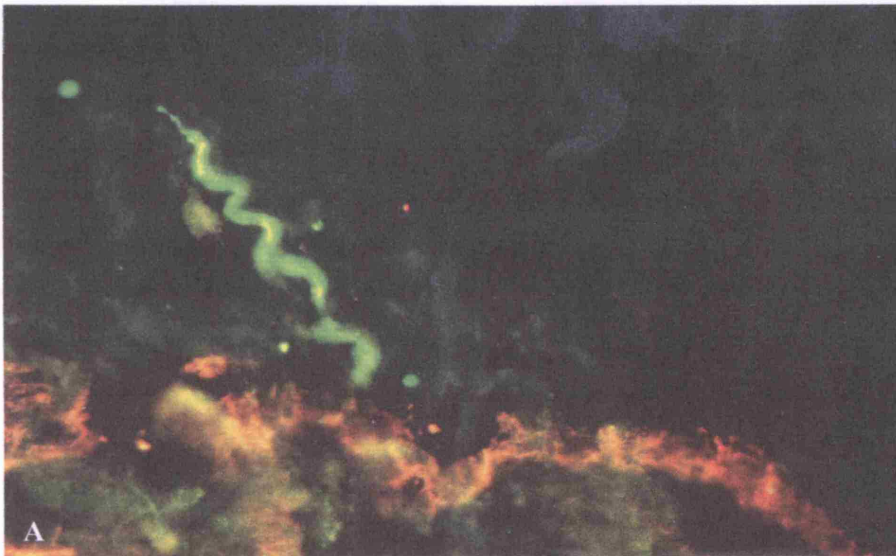


**Figure 4.3:** Extensive neural network within the wall of a blood vessel found in a human peritoneal adhesion. Note the circumferential and horizontal network of nerve fibres immunoreactive to calcitonin gene related peptide (CGRP) within the media (short arrow) and adventitial layer (long arrow) (x280).



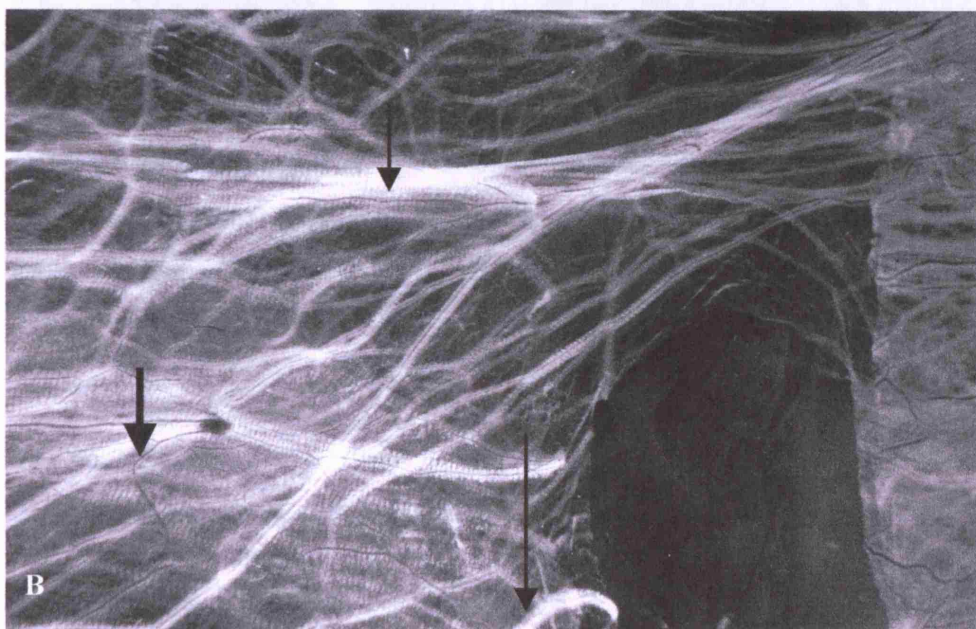
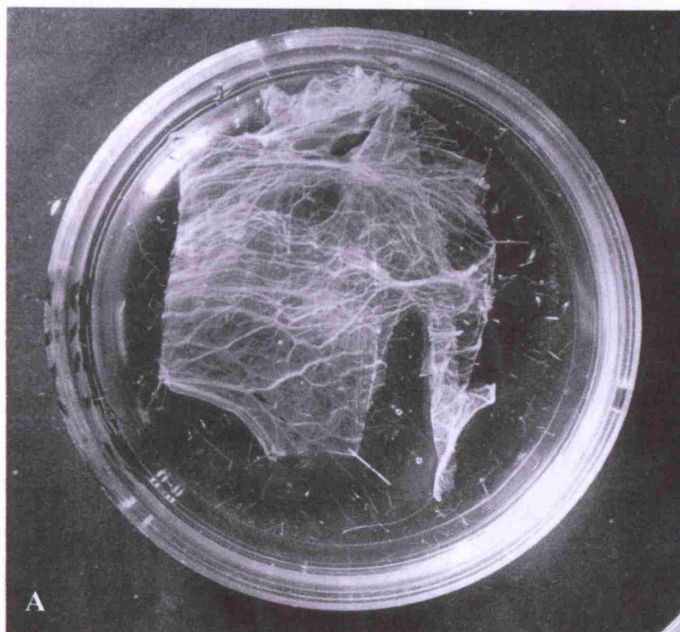


**Figure 4.4:** (A) Substance P-immunoreactive nerve fibres (green) with varicosities in human peritoneal adhesions. (B) Multiple blood vessels (red) immunoreactive for von Willebrand factor. (C) Co-localization of nerve fibres (green) with blood vessels (red). Some SP-IR nerve fibres appeared co-localised with BVs whereas others were independent of blood vessels (arrow). Bar represents 10  $\mu\text{m}$ .

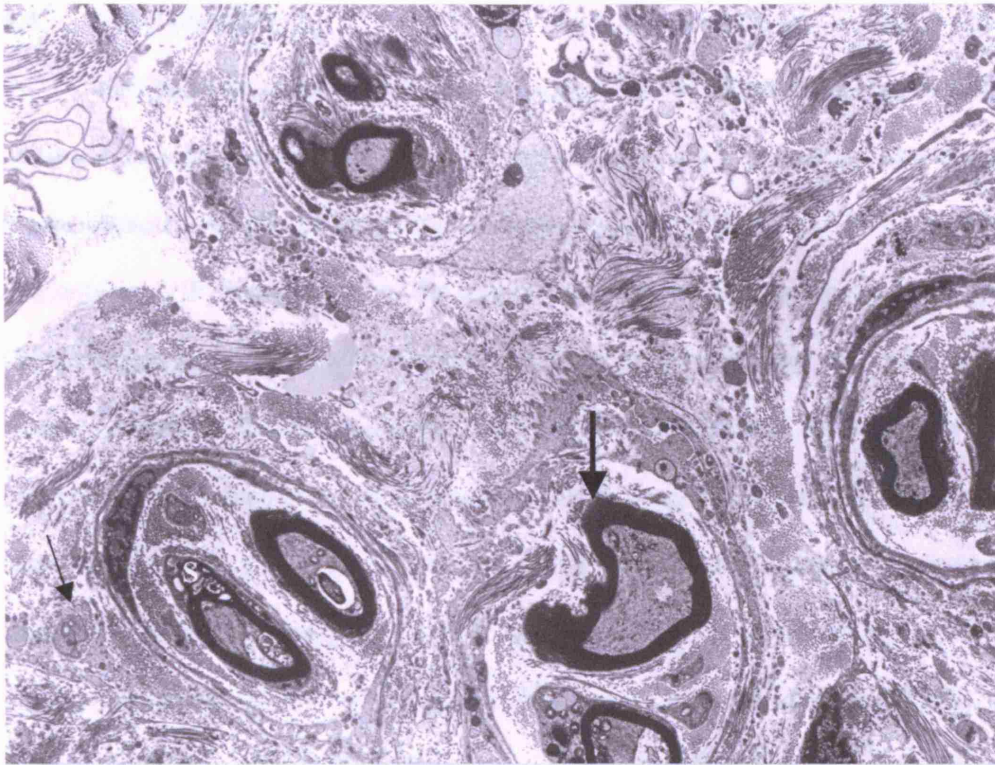


**Figure 4.5:** Co-localization of nerve fibres with blood vessels, in human peritoneal adhesion. (A): Synaptophysin immunoreactive nerve fibre (green) associated with a blood vessel immunoreactive for von Willebrand factor (red). (B): Tyrosine hydroxylase-immunoreactive nerve fibre (green) associated with a blood vessel-immunoreactive for von Willebrand factor (red). Bar represents 20  $\mu\text{m}$  for (A) and (B).





**Figure 4.6:** Distribution of blood vessels and nerve fibres in human peritoneal adhesions demonstrated in whole-mount preparations by acetylcholinesterase histochemistry (A) and higher power view in (B). Generally, nerve fibres (dark-coloured, short arrow) accompanied blood vessels (opaque, long arrow) arranged parallel to the long axis of adhesions, however some nerve fibres branched independently (thick arrow) (Ax5) (Bx18).



**Figure 4.7:** Transmission electron microscopy of a human adhesion showing myelinated (thick arrow) and non-myelinated nerve fibres (thin arrow) embedded within extracellular matrix. Schwann cells (S) are found surrounding the axons with collagen fibrils (x4800).

#### 4.4 DISCUSSION

This study has demonstrated the presence of nerve fibres in all human peritoneal adhesion samples examined. Serial sections from each specimen showed nerves immunoreactive to all the neuronal markers (sensory: CGRP and SP; autonomic: TH and enteric: VIP) irrespective of the site of the adhesion, its size or the estimated age. On ultrastructural examination the nerves consisted of myelinated and non-myelinated fibres. Substance P-immunoreactive and CGRP-immunoreactive nerve fibres were observed in the adhesion samples and because these peptides are abundant in sensory nerves it is likely that these fibres are sensory. The small-diameter non-myelinated fibres on electron microscopy could be C or A-delta fibres recognised as pain conducting fibres (Henry et al., 1982; Woolf and Weisenfeld-Hallin, 1986). These findings are comparable and similar to those demonstrated in previous study (Chapter 3) using murine adhesion model.

The results from this study highlight the limitations of previous studies, which showed the presence of nerves in a percentage of the human adhesions examined. Kligman and colleagues (1994) found nerve fibres in 58% of the pelvic adhesion specimens analysed immunohistochemically using a Schwann cell marker (S-100), whereas Tulandi and colleagues (1998) showed nerve fibres in 78% of pelvic adhesions examined using a neurofilament marker. Herrick et al. (2000) found that around 60% of the human peritoneal specimens examined histologically using Linder's silver nerve stain displayed nerves. This discrepancy is likely to be due to limitations in the techniques used in previous studies. In the current study, well-characterized antibodies against specific neuronal markers were used, and representative sections throughout the adhesion were stained.

The association between nerve fibres and blood vessels, as shown by dual immunolocalisation and acetylcholinesterase histochemistry is interesting and may

suggest that angiogenesis may play an important role in regulating the growth of nerve fibres in adhesions. Autonomic nerve fibres (Tyrosine hydroxylase and vasoactive intestinal peptide immunoreactive axons) usually accompany blood vessels and supply smooth muscle cells and glands. Hill and colleagues (1985) showed that after disruption of the nerve supply of the gut, re-innervation of arterial smooth muscle cells and intrinsic neurons of the gut was predominantly by sympathetic fibres (TH- and VIP-immunoreactive) regenerating from the interrupted paravascular nerve bundles. Sugitani and colleagues (1998) in a study of ileal transplantation, found that the initial regenerating fibres characterised with a number of neuronal markers such as CGRP, substance P and TH originated from the paravascular fibres in the mesentery and extended towards the anastomotic site. In the current study, there was generally an association between blood vessels and nerve fibres in terms of their orientation and proximity with both generally arranged along the longitudinal axis of peritoneal adhesion. New blood vessels have been found within developing peritoneal adhesions three days after trauma to serosal surfaces in animal studies (Milligan and Raftery, 1974). In particular, the current animal model (Chapter 2) showed blood vessels at day 3 and they were more abundant at day 5. Blood vessels may guide nerve growth through the release of trophic factors and the disposition of extracellular matrix tracts (Fu and Gordon, 1997). Therefore it may not be surprising that (TH-, VIP-, SP-, CGRP-, and Synaptophysin-IR) nerve fibres were observed in adhesion samples accompanying blood vessels. However isolated nerve fibres that appeared independent of blood vessels also suggests that their growth may be regulated by other stimuli such as chemotactic inflammatory mediators, or inhibited by dense fibrotic areas within the adhesions.

The effect of innervation on adhesion formation and maturation is not known, although it is thought that neuropeptides released from nerve fibres are involved in a number of processes. For example, sensory and autonomic nerves interact with blood

vessels to regulate blood flow neurogenic inflammation (Sternini, 1997). Both neuropeptides, neurokinin A and substance P have been shown to stimulate fibroblast and arterial smooth muscle cell proliferation, and neurokinin A also induces fibroblast chemotaxis (Nilsson et al., 1985; Harrison et al., 1995) while substance P causes vasodilatation and stimulates histamine secretion leading to increased capillary permeability (Dockray et al., 1986). Furthermore, Calcitonin gene-related peptide and substance P are thought to be essential for normal wound healing. Denervated skin wounds heal more slowly and have a reduced inflammatory infiltrate compared with control wounds (Richardson et al., 1997). Hence repeated distortion and stretching of innervated adhesions may lead to the release of neuropeptides and so influence fibroblast activity and modulate the deposition of ECM during adhesion maturation. The combination of stretching and innervation may play a role in the continual remodelling of adhesions (Herrick et al., 2000).

The presence of nerves in peritoneal adhesions may also be involved in the facilitation of pain conduction. This study is the first demonstration of sensory nerve fibres including those that may generate pain stimuli (CGRP- and substance P-IR fibres), in human peritoneal adhesions. However, five patients reported no abdominal/pelvic pain 3 to 6 months pre-operatively even though their adhesions contained non-myelinated axons and fibres immunoreactive for the sensory neuronal markers known to be present in pain conducting fibres. This suggests that the presence of sensory nerves in adhesions does not predispose a patient to chronic abdomino-pelvic pain. The nerves may be non-functional or require a series of inducing stimuli to transmit pain sensations as in visceral peritoneum (Cervero, 1999). The number of C primary and A-delta afferent nerve fibres supplying the viscera is small in comparison with the number of fine afferents of somatic nerves to the abdominal wall (Janig, 1996). These visceral afferents unlike somatic fibres, probably do not have the nociceptive function and the high threshold of those specialized nerve endings that when stimulated specifically result in the sensation of pain. Instead, they terminate in mechanoreceptors capable of a graded response that depends on the intensity of the stimulus. Visceral pain

is thought to reflect an abnormal involvement of the neural mechanisms usually concerned with the mediation of reflexes and amorphous sensations, which with added mechanical or chemical stimuli respond with increased intensity and therefore perceived as painful stimuli. A similar mechanism may exist in human adhesions where a combination of distension, hypoxia and inflammation may result in the stimulation of sensory fibres in the adhesions and the perception of chronic abdomino-pelvic pain. In addition to this direct role adhesions may cause abdomino-pelvic pain by restricting the mobility of internal organs and thus stimulating pre-existing stretch sensitive fibres in the muscle layer of the adjoining viscera (Lundberg et al., 1973; Kresch et al., 1984; Keltz et al., 1995). This area remains controversial and highly complex and will require detailed and thorough experimental studies to definitely confirm an involvement of adhesions in chronic pain.

In conclusion, this study has shown the presence of sensory nerves within the human peritoneal adhesions specimens from all patients. Although pain-physiological studies were not conducted, it is possible that the thin non-myelinated sensory fibres observed, are capable of conducting pain stimuli. However, not all patients in this study experienced chronic pelvic pain; therefore, although all adhesions may be able directly to give rise to pain sensations, there are likely to be other factors to consider, in addition to innervation, such as peritoneal pathology, organ mobility and psycho-somatic manifestations. Furthermore, a quantification study was not performed, therefore there may be a different number of sensory fibres present in different patients with or without associated abdominal pain.

## **CHAPTER FIVE**

### **THE ROLE OF FIBRINOLYSIS IN MURINE ADHESION FORMATION**



## 5.1 INTRODUCTION

Peritoneal adhesions are formed when closely opposed surfaces of the parietal or visceral peritoneum are damaged and the mesothelial layer is removed to expose the underlying tissue. Disruption of the protective mesothelial layer of the peritoneum is thought to cause a local inflammatory response leading to the formation of fibrin-rich exudate (fibrinous adhesion) as part of the haemostatic process. The deposition of fibrin between the damaged surfaces is an essential component of normal tissue repair. These filmy fibrinous adhesions may be transient and degraded by proteases of the fibrinolytic system within a few days of injury. Normal restoration of the peritoneal surface with re-epithelialization by mesothelial cells then occurs (Jackson, 1958; Milligan and Raftery, 1974).

Persistence of fibrin, presumably due to an imbalance between fibrin deposition and clearance by the fibrinolytic activity of the peritoneum (Holmdahl, 1997), results in the remaining fibrinous deposits becoming organized into permanent fibrous adhesions. This is characterized by an invasion of fibroblasts and endothelial cells leading to collagen deposition and vascularization as shown in Chapter 2. Therefore, it may be feasible that the restoration of fibrinolytic activity during the critical period of peritoneal healing could prevent, or at least diminish adhesion formation.

The normal peritoneum has inherent fibrinolytic activity mainly derived from surface mesothelial cells. In 1969, Myhre-Jensen and colleagues suggested that mesothelial cells activate plasminogen through the production of plasminogen activators tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA) and that the plasminogen activating activity of the peritoneum determines whether fibrinolysis or organisation of fibrinous adhesions follows inflammation. Peritoneal fibrinolytic activity have been shown to be suppressed in both experimental animal studies and in humans by trauma, infection, ischaemia or thermal injury at the site of injury resulting



in decreased levels of tPA, increased fibrinolytic inhibitors PAI-1 and PAI-2, both during and immediately post-operatively (Buckman et al., 1976; Raftery et al., 1981; Vispond et al., 1990; Whawell et al., 1993; Holmdahl, 1997). Fibrinolytic activity has been shown to rise 3 days after peritoneal trauma and increase to a maximum at day 8 in a rat model (Pados et al., 1992). Gervin et al. (1973) demonstrated basal fibrinolytic activity in all the gastrointestinal serosa after injury in a dog model, and if fibrinolytic activity was decreased more than 50%, then adhesion formation occurred. However, the use of intraperitoneal urokinase in those animals with 50% or greater reduction of fibrinolytic activity completely prevented adhesion formation in 80% of the animals.

The role of each of the plasminogen activators (tPA and uPA) in preventing the formation of peritoneal adhesions following various insults is unclear. Traditionally, tPA is viewed as a protease involved in clearance of intra-vascular fibrin, whereas uPA is involved with cell migration as it is cell surface bound. To elucidate the role of each of the plasminogen activator in adhesion formation we have taken the novel approach of using mice deficient in these proteases. Genetic manipulation of the plasminogen activator system in knockout mice provides a novel experimental approach to study the physiological and pathological roles of each of these proteases following various experimental conditions. This has not only provided a unique opportunity to determine the physiological function or phenotype of a mutation of each PA gene product in embryonic and postnatal development, but has also enabled the investigator to create animal models for human genetic disorders including atherosclerosis, glomerulonephritis or malignancy. Mice deficient in tPA and uPA show different phenotypes. They both appeared normal and have a normal life-expectancy, however, u-PA deficient mice occasionally developed rectal prolapse and chronic non-healing ulcers with excessive deposition of fibrin in the liver, intestines, lungs, gonads and kidneys (Carmeliet et al., 1994). Mice deficient in tPA have a reduced thrombolytic potential

and increased incidence of endotoxin-induced thrombosis (Kluft, 1988). Mice with a combined deficiency of tPA and uPA suffer significant growth retardation, markedly reduced infertility and shorter life expectancy with more extensive fibrin deposition than in mice with single PA deficiency (Carmeliet et al., 1994).

The aim of this study was: (a) to assess peritoneal adhesion formation following serosal trauma in uPA and tPA deficient mice and compare the findings with those in wild-type mice using our modified surgical adhesion model and (b) to assess adhesion formation in these animals using a non-surgical approach comprising of an acute or chronic inflammatory approaches. Adhesions were assessed macroscopically at set times post-injury and peritoneal cell infiltrate was analysed following an inflammatory stimulus. It is generally accepted that adhesions are permanent after 7 days following a surgical injury in all species assessed, however, it is not clear when adhesions become permanent after an inflammatory insult, therefore multiple time points were analysed.

## 5.2 MATERIALS AND METHODS

Before these studies started, Home Office licence requirements were fulfilled for the project and personal use.

### 5.2.1 Animals

Mice deficient in tPA ( $tPA^{-/-}$ ) or uPA ( $uPA^{-/-}$ ) and wild type (WT) littermates on a mixed genetic background (75%C57/BL6), were established through collaboration with Professor Carmeliet, Leuven University, Belgium. Breeding in controlled conditions of temperature and light and with water and pellet food ad libitum was performed in the Biological Services Department, University College London (UCL). The genotype of the mice was confirmed by RT-PCR using set primers (L. Dawson and A. De Giorgio-Millar, Department of Medicine, UCL. A total of 210 male and female mice deficient in  $uPA^{-/-}$ ,  $tPA^{-/-}$  and wild-type (WT-PA), 8-12 weeks old with average weight of 25g were used throughout this study. The temporal and spatial formation of adhesions was assessed following both surgical and inflammatory insults using a variety of experimental models. Uninjured mice from all 3 strains were not found to show any evidence of adhesion formation before the study commenced.

### 5.2.2 Experimental animal models

**Trauma model:** A total of 58 mice were used:  $tPA^{-/-}$  ( $n = 19$ );  $uPA^{-/-}$  ( $n = 20$ ) and WT ( $n = 19$ ). The model involved a standardised trauma to the caecal surface and adjacent abdominal body wall combined with haemorrhage from a damaged mesenteric vessel as described in Chapter 2. Our previous studies suggested that 100% adhesions formation occurred after the two serosal surfaces were apposed with stitches and various combinations of these procedures resulted in less animals forming adhesions. Therefore, opposition of the damaged surfaces was omitted to be able to demonstrate an increase as well as decrease in the number of animals developing adhesions. Under general anaesthetic, and through a midline laparotomy incision, a standardised injury was

performed by scrapping specific areas of the opposing surfaces of the caecum and abdominal wall, followed by trauma to mesenteric vessel as described in Chapter 2. Assessment of adhesion formation was performed macroscopically on day 7 post-surgery. Each mouse was classified as positive if it had developed one or more peritoneal adhesions. Using this model, adhesions were further divided into category 1, if found at the traumatised site (between the caecum and body wall), category 2, if between one of the injured serosal surfaces and an uninvolved site and category 3, if between uninvolved serosal surfaces including the midline incision. Adhesions found at the trauma site were collected for histological analysis as described in Chapter 2.

**Acute inflammatory model:** This model was based on non-lethal inflammatory insults produced by intra-peritoneal injection of 4% Brewers Thioglycollate (TG; Difco, East Molesey, UK) in 1ml PBS pH 7.4, producing an acute self-limiting sterile peritonitis resolving over ten to twelve days (Bellingan et al., 1996). A total of 46 animals were used: tPA<sup>-/-</sup> (n = 15); uPA<sup>-/-</sup> (n = 15) and WT (n = 16). The animals were sacrificed by using carbon dioxide (CO<sub>2</sub>) over 5 minutes at set time points: day 5 (n = 4 per strain), day 7 (n = 7/8 per strain), day 9 (n = 4 per strain) and peritoneal lavage performed. Macroscopic examination of peritoneal cavity categorised adhesions as for the surgical model.

**Chronic inflammatory model:** Mice of each strain: t-PA<sup>-/-</sup> (n = 35); u-PA<sup>-/-</sup> (n = 36) and WT (n = 35) were injected intra-peritoneally with 100mg/kg heat-inactivated *Corynebacterium parvum* (*C. parvum*) in 1 ml PBS pH 7.4 leading to chronic peritonitis with hepatosplenomegaly and ascites. Animals were sacrificed by CO<sub>2</sub> as above. After peritoneal lavage, formation of adhesion was assessed at set time points: 1 week (n = 8 per strain), 2 weeks (n = 12 per strain), 3 weeks (n = 11/12 per strain) and 4 weeks (n = 4 per strain).

### **5.2.3 Peritoneal lavage and inflammatory cell profile**

After killing the animals, 4ml of ice-cold sterile PBS, pH 7.4 was injected intra-peritoneally. After 2 minutes of gentle abdominal massage to equilibrate the fluid, the peritoneal wash was collected for cell count. Fluid for the cell count (~ 400 µl) was collected into pre-chilled eppendorf to which 10% fetal calf serum (final) was added to help maintain cell integrity. Total nucleated peritoneal fluid cell count was determined after staining viable cells with trypan blue (Sigma Chemical Co., Poole, UK) using a 'Neubaur improved' hemacytometer (Assistant, Hecht KG, Germany), with counts expressed as the number of cells per ml of PBS lavage fluid.

### **5.2.4 Statistics**

Adhesions were scored as present or absent with (1) denoting that a single adhesion was found, (2) as two adhesions were found, irrespective of category. For statistical analysis, the Kruskal-Wallis non-parametric analysis was performed. Statistical significance was defined as  $p \leq 0.05$ , with mean number of adhesions per mouse (mean $\pm$  SEM) compared with wild type or the alternate PA deficient strain.

## 5.3 RESULTS

### 5.3.1 Surgical model

All the animals used in this study survived the experimental procedures and suffered no ill effects. Seven days after surgical trauma, only a few mice in any group developed adhesions at the trauma site (category 1), whereas the majority of adhesions were found to involve one injured surface and an uninvolved site, such as caecum to small bowel (category 2), or to be totally independent of the original injury site for example, midline incision to pelvic fat body (category 3) (Figure 5.1). Several mice developed multiple adhesions of more than one category (adhesion mass) and this appeared to be more prevalent in mice from the  $tPA^{-/-}$  group (figure 5.2). In general, more adhesions were formed by  $tPA^{-/-}$  mice at day 7 post-operatively (15/19) than in the  $uPA^{-/-}$  (10/20) or WT (10/19) groups. In addition, the mean number of adhesions per mouse was highest in the  $tPA^{-/-}$  group compared with the other two groups ( $tPA^{-/-}$ ,  $1.63 \pm 0.26$ ;  $uPA^{-/-}$ ,  $1.05 \pm 0.28$ ; WT,  $1.0 \pm 0.25$ ), however there was no statistically significant difference between the groups ( $p > 0.05$ ).

### 5.3.2 Acute inflammatory model

All mice used in this study survived the experimental procedures and suffered no ill health. An intra-peritoneal injection of 4% Brewer's Thioglycollate provoked a pronounced increase in peritoneal lavage cells in all three groups 5 days after injection compared with baseline levels. This was significantly increased in the  $tPA^{-/-}$  group compared with  $uPA^{-/-}$  ( $p < 0.05$ ) and WT ( $p < 0.05$ ) groups (Figure 5.3B). All mice had developed one or more adhesions at this time, generally between the lobes of the liver, the omentum, and pelvic fat bodies and adjacent organs.

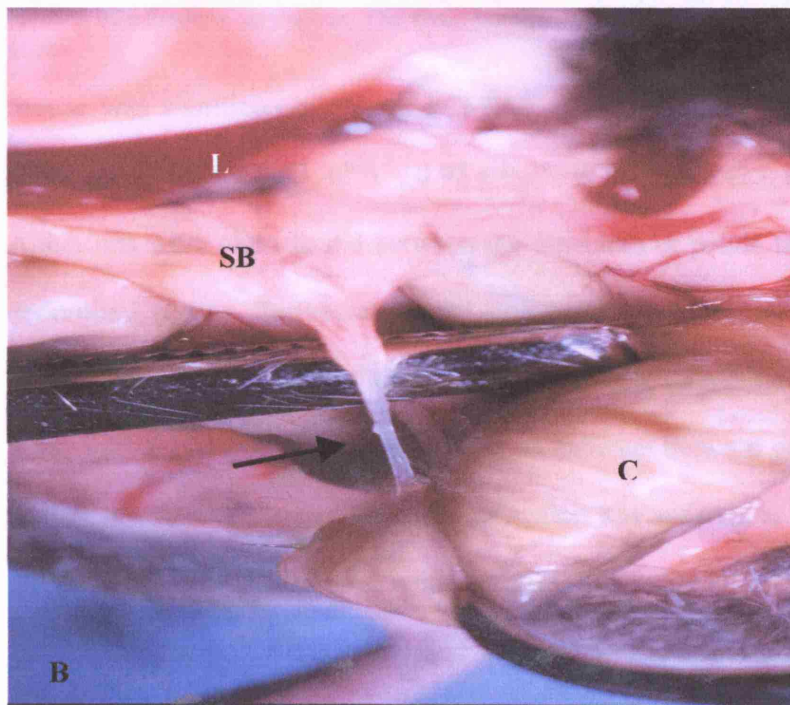
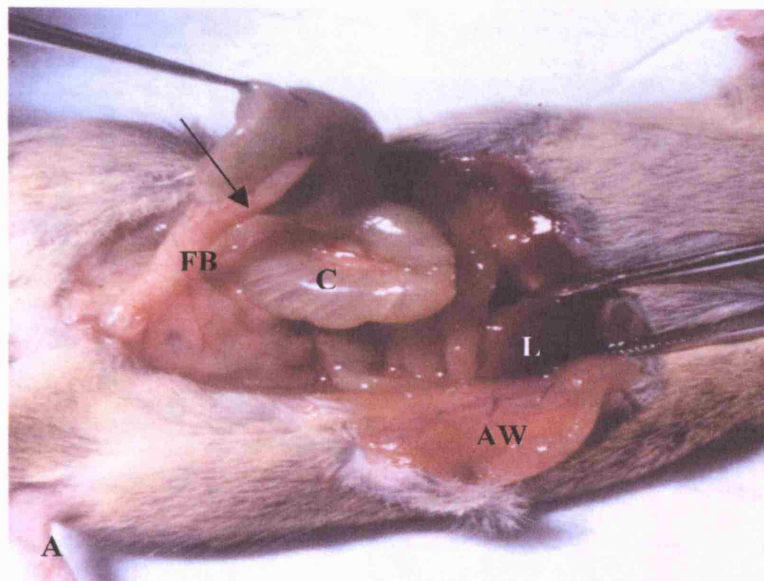
All adhesions at day 5 were thin and filmy in nature. By contrast, on day 7 post-injection, adhesions were only found in only one or two mice in each group ( $tPA^{-/-}$

<sup>-/-</sup> 2/7; uPA<sup>-/-</sup> 1/7; WT, 1/8). The mean number of adhesions per mouse at day 5 (t PA<sup>-/-</sup>  $1.5 \pm 0.5$ ; u PA<sup>-/-</sup>  $1.5 \pm 0.6$ ; WT  $1.5 \pm 2.9$ ) and day 7 (t PA<sup>-/-</sup>  $0.28 \pm 0.18$ ; u PA<sup>-/-</sup>  $0.14 \pm 0.14$ ; WT  $0.125 \pm 0.125$ ) was similar between groups (Figure 5.3A), and there was no statistically significant difference between the groups at either time group (WT vs t PA<sup>-/-</sup>  $p > 0.05$ ; WT vs u PA<sup>-/-</sup>  $P > 0.05$ ; u PA<sup>-/-</sup> vs t PA<sup>-/-</sup>  $p > 0.05$ ).

By day 7, intra-peritoneal lavage cells had substantially reduced apart from in the uPA<sup>-/-</sup> group in which the number of inflammatory cells remained significantly elevated compared with the other two groups (u PA<sup>-/-</sup> vs WT  $p < 0.05$ ; u PA<sup>-/-</sup> vs t PA<sup>-/-</sup>  $p < 0.05$ ; Figure 5.3A). On day 9 post-injection, no adhesions were found in any of the mice and inflammation had returned to baseline levels in tPA<sup>-/-</sup> and WT mice whereas a significant inflammatory response remained in the uPA<sup>-/-</sup> mice (u PA<sup>-/-</sup> vs WT  $p < 0.05$ ; u PA<sup>-/-</sup> vs t PA<sup>-/-</sup>  $p < 0.05$ ). In summary, there was no statistically significant difference in the mean number of adhesions between any of the groups at any time point.

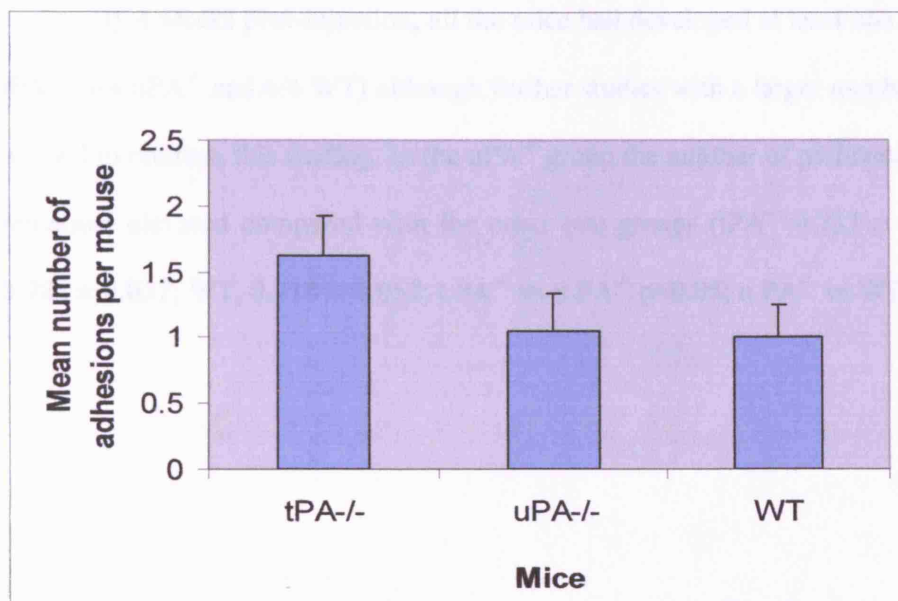
### 5.3.3 Chronic inflammatory model

All mice used in this study survived the experimental procedures and suffered no ill health. Following peritoneal injection of heat-inactivated *C. parvum*, there was no adhesion formation in any of the mice at 1 week, although there was evidence of tabes mesentica (inflammatory response and enlargement of draining mesenteric lymph nodes), pancreatitis, hepatosplenomegaly and inflammatory nodules on the liver. Number of peritoneal lavage cells had increased compared with baseline control values, but was similar in all three groups of mice (Baseline control values: tPA<sup>-/-</sup>  $0.92 \pm 0.72$ ; uPA<sup>-/-</sup>,  $0.996 \pm 0.06$ ; WT,  $0.994 \pm 0.083$ . One week: t PA<sup>-/-</sup>  $1.81 \pm 0.09$ ; uPA<sup>-/-</sup>,  $1.64 \pm 0.09$ ; WT,  $1.64 \pm 0.09$ ; Figure 5.4B). However by 2 weeks, more of both tPA-deficient mice had developed adhesions compared with the WT group (tPA<sup>-/-</sup>, 9/12; uPA<sup>-/-</sup>, 7/12; WT, 3/12) (Figure 5.4A). At this time, the mean number of adhesions per



**Figure 5.1:** Post-operative peritoneal adhesion formation one-week after surgery in  $tPA^{-/-}$  mice. (A): Pelvic fat body (arrow) adherent to the caecum (category 2). (B): Peritoneal adhesion (arrow) spanning between the small bowel and caecum (category 2). C: caecum; L: liver; SB: small bowel; AW: abdominal wall; FB: Pelvic fat body.





**Figure 5.2:** Adhesion formation following surgery at day 7 post-operatively. Data represents the mean number of adhesions per mouse. The mean number of adhesions per mouse was highest in the tPA<sup>-/-</sup> group

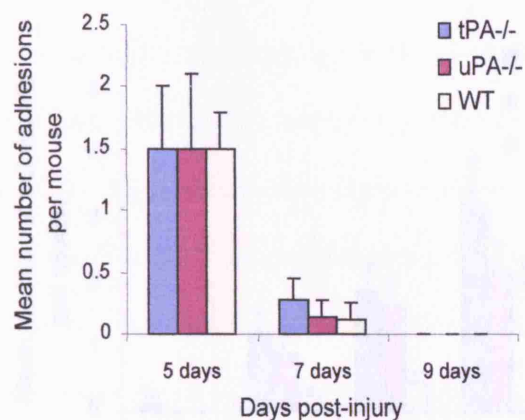
mouse was significantly greater in t-PA<sup>-/-</sup> group compared with WT but not u PA<sup>-/-</sup> group (tPA<sup>-/-</sup>  $1.25 \pm 0.33$ ; uPA<sup>-/-</sup>,  $0.95 \pm 0.25$ ; WT,  $2.5 \pm 0.125$ ; t PA<sup>-/-</sup> vs WT  $p < 0.05$ ).

Adhesions were often found between the omentum or pelvic fat bodies and the upper abdominal organs. Peritoneal lavage cell number was substantially increased in all groups compared with baseline pre-injection levels (tPA<sup>-/-</sup>  $2.4 \pm 0.04$ ; uPA<sup>-/-</sup>,  $2.39 \pm 0.03$ ; WT,  $2.19 \pm 0.04$ ) but not significantly different between groups ( $p > 0.05$ ).

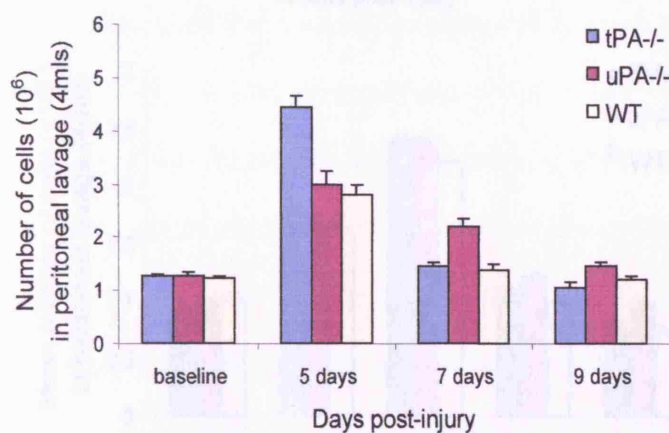
A similar pattern of adhesion formation was apparent at 3 weeks post-injection, with a substantially increased number of mice showing adhesions in the PA-deficient groups (8/11 tPA<sup>-/-</sup>, 8/12 uPA<sup>-/-</sup> and 4/11 WT). The mean number of adhesions per mouse was significantly greater in t-PA<sup>-/-</sup> and uPA<sup>-/-</sup> group compared with WT group (tPA<sup>-/-</sup>  $1.91 \pm 0.41$ ; uPA<sup>-/-</sup>,  $1.67 \pm 0.43$ ; WT,  $0.45 \pm 0.197$ ; t PA<sup>-/-</sup> vs WT  $p < 0.05$ ; u PA<sup>-/-</sup> vs WT  $p < 0.05$ ). At this time, inflammation had substantially reduced apart from in the uPA<sup>-/-</sup> group in which the number of peritoneal lavage cells remained significantly elevated compared with the other two groups (tPA<sup>-/-</sup>  $1.07 \pm 0.04$ ; uPA<sup>-/-</sup>,  $1.21 \pm 0.04$ ; WT,  $0.983 \pm 0.05$ ; t PA<sup>-/-</sup> vs u PA<sup>-/-</sup>  $p < 0.05$ ; u PA<sup>-/-</sup> vs WT  $p < 0.05$ ).

By 4 weeks post-injection, all the mice had developed at least one adhesion (4/4 tPA<sup>-/-</sup>, 4/4 uPA<sup>-/-</sup> and 4/4 WT) although further studies with a larger number of mice are needed to confirm this finding. In the uPA<sup>-/-</sup> group the number of peritoneal lavage cells remained elevated compared with the other two groups (tPA<sup>-/-</sup>  $0.783 \pm 0.062$ ; uPA<sup>-/-</sup>,  $0.984 \pm 0.037$ ; WT,  $0.718 \pm 0.052$ ; tPA<sup>-/-</sup> vs uPA<sup>-/-</sup>  $p < 0.05$ ; uPA<sup>-/-</sup> vs WT  $p < 0.05$ ).

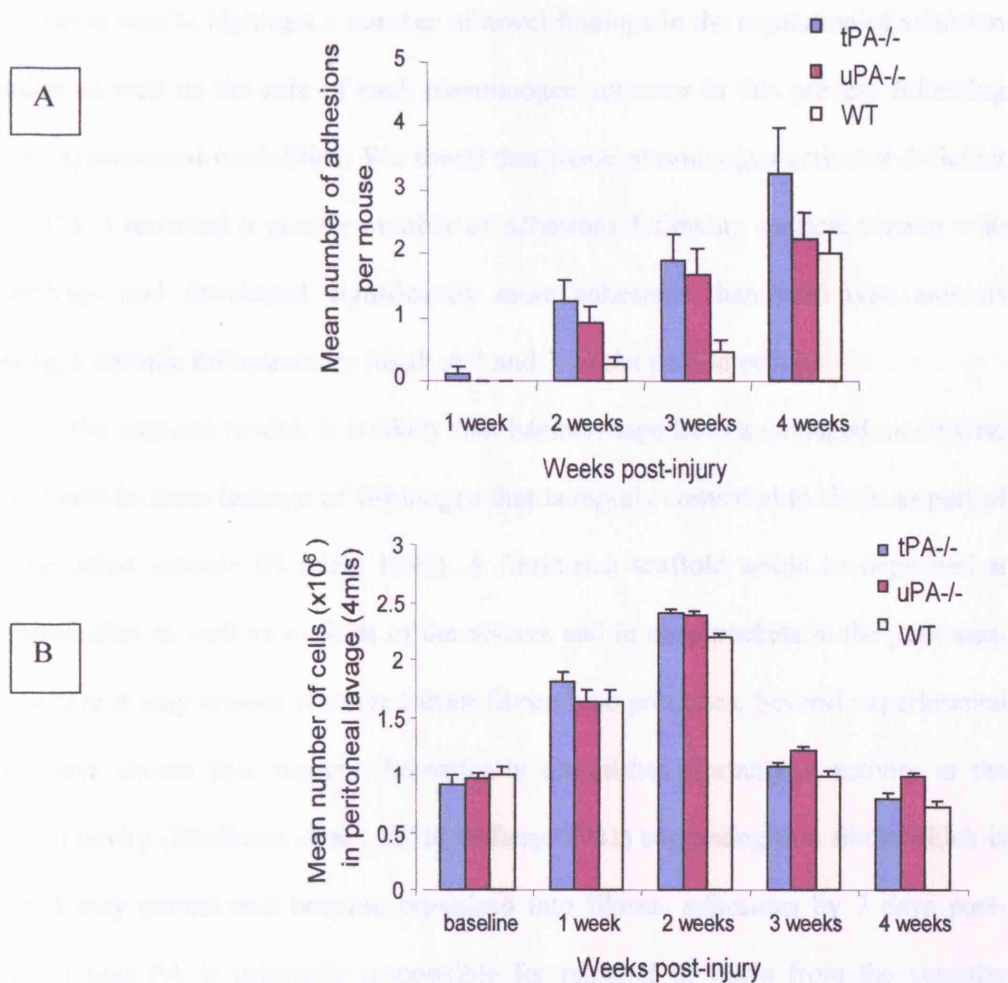
A



B



**Figure 5.3:** Adhesion formation following acute inflammatory insult in all strains. (A) Mean number of adhesions per mouse per group. (B) Number of cells in peritoneal lavage. Data expressed as means  $\pm$  SEM.



**Figure 5.4:** Adhesion formation following chronic inflammatory insult in all strains. (A) Mean number of adhesions per mouse per group. (B) Number of cells in peritoneal lavage. Data expressed as means  $\pm$  SEM. Tissue plasminogen activator-deficient mice developed significantly more adhesions two weeks after i.p injection of *C. parvum* compared with urokinase-deficient and wild type control mice. ( $p < 0.05$ , Kruskal-Wallis). Inflammatory cellular response was increased compared with baseline control values up to 4 weeks post-injection where the cell profile returned to baseline levels in the t-PA-/- and WT but remained elevated in the uPA-/-.

## DISCUSSION

These results highlight a number of novel findings in the regulation of adhesion formation as well as the role of each plasminogen activator in this process following various experimental modalities. We found that tissue plasminogen activator deficient mice (t-PA<sup>-/-</sup>) recorded a greater number of adhesions following surgical trauma with haemorrhage and developed significantly more adhesions than wild type animals following a chronic inflammatory insult at 2 and 3 weeks post-injection.

In the surgical model, it is likely that haemorrhage from a damaged mesenteric vessel results in mass leakage of fibrinogen that is rapidly converted to fibrin as part of the coagulation cascade (Herrick, 1996). A fibrin-rich scaffold would be deposited at the injured sites as well as in folds of the viscera and in deep pockets in the peritoneal cavity where it may remain protected from fibrinolytic proteases. Several experimental studies have shown that surgery dramatically diminishes fibrinolytic activity in the peritoneal cavity (Buckman et al., 1976; Raftery, 1981) suggesting that fibrin which is deposited may persist and become organised into fibrous adhesions by 7 days post-surgery. Tissue-PA is primarily responsible for removal of fibrin from the vascular system through its specific affinity for fibrin (Collen & Lijnen, 1991) and mice lacking t-PA have a significantly reduced thrombolytic potential (Carmeliet et al., 1994). Therefore, these mice are probably recording more adhesions following this procedure due to insufficient fibrinolytic activity from a combination of surgery and a lack of t-PA. Carmeliet and colleagues (1994) showed that there was no compensatory activity of either PA in the correspondingly deficient mouse suggesting that t-PA is essential for the prevention of fibrous adhesion formation in this model and that the presence of u-PA or any other fibrinolytic protease was not sufficient to compensate the lack of t-PA.

Acute inflammation is likely to cause a mild peritoneal injury resulting in extravascular fibrinogen leakage from inflamed and damaged peritoneal vessels as well

as in direct binding of fibrinogen derived from peritoneal fluid to areas of damaged serosa. With this inflammatory model, all mice developed fibrinous adhesions initially but these were transient and degraded within 9 days, suggesting that both groups of PA-deficient mice had sufficient fibrinolytic activity to degrade any fibrin deposited before it was organised. This activity is likely to be due to the complementary PA or another fibrinolytic protease, such as neutrophil elastase and/or members of the matrix metalloprotease family. Adhesions were mainly found between lobes of the liver. This is an extremely well vascularised organ with naturally opposed serosal surfaces and so with sites protected from fibrinolytic activity. Omental and pelvic fat body adhesions were also common although, as these structures are extremely adhesive and may associate with injured serosal surfaces within a few hours. Furthermore, their development may be regulated by a different mechanism not reliant on reduced fibrinolysis (Wilkosz et al., 2005). All the adhesions observed using this acute inflammatory model were thin and filmy in nature and were not organised into true persisting collagenous structures after five days.

In the chronic inflammatory model, peritoneal lavage cellularity was maximal at two weeks after intra-peritoneal injection and this corresponded to a greater number of both tPA- and uPA- deficient animals developing adhesions compared with wild-type animals and this was significant for t PA-deficient mice at 2 weeks and both t PA and u PA-deficient animals at 3 weeks. Chronic inflammation is likely to cause a substantial amount of tissue damage with increased fibrin deposition creating areas of ischaemia and loss of the protective mesothelial layer more severe than acute inflammation (Bellingan et al., 1996). Our results suggest that both tPA and uPA are required to clear fibrin 2 weeks following a chronic inflammatory insult. Macrophages are primarily responsible for the clearance of extravascular fibrin and are thought to require uPA for pericellular proteolysis and cell migration (Haney, 2000 b). Indeed, Carmeliet and

colleagues (1994) found that macrophages derived from uPA-deficient mice showed impaired fibrin dissolution and migration compared with those obtained from tPA-deficient and WT mice. Furthermore, these may be secondary effects involving decreased activation of MMP's by uPA leading to further reduction of fibrinolysis. The current study clearly demonstrates that tPA is essential in regulating plasmin mediated fibrin degradation and preventing permanent adhesion formation in this model. It is always thought that mature adhesion always form by 7 days post-injury, but this model suggests it may take longer with ongoing event over several weeks that will need further investigation. Dual tPA and uPA deficient mice are more likely to develop spontaneous adhesions.

Several studies have suggested that peritonitis and ongoing pelvic inflammatory disease cause a reduction in fibrinolytic activity mainly due to a significant increase in PAI levels (Whawell et al., 1993; van Goor et al., 1994). Although we did not measure levels of PAI in our study, a substantial enhancement in PA inhibition might explain the lack of compensatory activity of the complementary PA with the development of adhesions in all strains including WT by 4 weeks post-injection.

In conclusion, both plasminogen activators appear to play a role in preventing the formation of permanent peritoneal adhesions following a chronic inflammatory episode. The contribution of each protease is likely to relate to the site and type and duration of injury and the physical properties of the fibrinous exudate produced. Several studies have used fibrinolytic agents in adhesion prevention with positive results (Hellebrekers et al., 2000). This seems to be a promising approach for the future, but further research needs to be undertaken to validate its significance before its application in clinical practice.

**CHAPTER SIX**  
**OVERALL DISCUSSION**



The clinical significance of post-operative peritoneal adhesions and their great impact on patients' welfare and health service resources is obvious and can not be over emphasised. Adhesions occur in 73-100% of patients undergoing laparotomies leading to subsequent complications including infertility in women, intestinal obstruction and possibly chronic abdominal pain (Menzies, 1992). The present study in Chapter 2 monitored the formation of intra-peritoneal adhesions in a murine model and showed that adhesion formation was inevitable following a number of consecutive operative procedures. Similar findings were observed by Weibel and Majno (1973) with human subjects. A multiple injury approach was needed to produce a consistent reproducible standardized post-operative peritoneal adhesion model that could be used to elucidate mechanisms involved in adhesion formation in greater details from previous studies.

A number of novel findings resulted from this study. For example, (1) The presence of fibrin threads 3 days post-operatively in areas distant from the injury site, reflects the generalised effect of localised trauma within the whole peritoneal cavity and may explain why adhesions sometimes form at areas that were not injured directly. (2) The abundance of inflammatory cells surrounding suture material several weeks post-surgery, highlights the intensity of inflammatory response associated with foreign body reaction and possible involvement in subsequent adhesion maturation. (3) The presence of omental fenestrations associated with inflammatory cells and the ability of the omentum and pelvic fat body to rapidly adhere to damaged serosa is consistent with the function of the omentum acting as "policeman of the abdomen". Future studies should address the mechanisms whereby this adipose rich tissue adheres to sites of trauma and whether fenestrations play a role as suggested by Ryan et al. (1973) by acting as a sponge. (4) Lastly, the finding of detachment of the muscular layer of the caecal wall from the submucosa at 3 weeks post-operatively was interesting and suggests either

contraction of regenerating muscle fibres or contraction of the adhesion possibly by myofibroblasts.

Ultrastructural findings from Chapter 2 also provided the first direct evidence that nerve fibres were present in murine adhesion and this led to a more detailed study described in Chapter 3. Nerve fibres immunoreactive for synaptophysin, calcitonin gene related peptide (CGRP), substance P (SP), vasoactive intestinal peptide (VIP) and tyrosine hydroxylase (TH) were observed within adhesions, 2 weeks post-surgery and appeared to originate from the caecal wall. Acetylcholinesterase histochemistry clearly demonstrated that nerve fibres spanned the length of the adhesion with the majority associated, but some were not associated with blood vessels. Furthermore, ultrastructural analysis showed that these fibres contained both myelinated and non-myelinated axons, the latter appearing thinner in diameter suggesting that they may be C or A- $\delta$  fibres, many of which are known to be pain-conducting fibres (Torotoro and Grabowski, 1996). (Findings were published Sulaiman et al., Journal of Pathology, 2001 and a copy enclosed at end of MD thesis).

Chapter 4 showed that all human peritoneal adhesion samples examined demonstrated the presence of nerve fibres that were also immunoreactive to all the neuronal markers (sensory, autonomic and enteric) previously analysed in mice irrespective of the site of the adhesion, its size or estimated age (Findings published Sulaiman et al., Annals of Surgery, 2002 and a copy enclosed at the end of MD thesis). Furthermore, past surgical intervention, inflammation or reports of the presence or absence of chronic pelvic pain did not seem to influence the distribution or type of nerve fibre detected. This raises the possibility that a similar mechanism of nerve ingrowth occurs in human adhesions as in mice irrespective of whether adhesions form due to infection or surgery. The presence of non-myelinated fibres and Substance P and CGRP immunoreactive nerve fibres also suggests that human adhesions may be capable of

conducting pain sensation. Adhesions may also be indirectly involved in the aetiology of chronic abdominal pain sensation, by restricting the mobility of adjacent organs and so cause an inordinate degree of stretching of the smooth muscle and thus presumably some pain.

The role of fibrinolysis in adhesion formation was assessed in Chapter 5, and results showed that tissue plasminogen activator deficient mice were more susceptible to adhesion formation following surgical trauma and a chronic inflammatory insult than urokinase plasminogen activator deficient and wild type animals. (These findings were published Sulaiman et al., Biochemical Society Transactions, 2002 and a copy is enclosed at the end of the thesis). This study shows the importance of tPA in clearance of fibrin in peritoneal cavity.

Future studies are needed focusing on different aspects of adhesion formation to increase our understanding of its pathophysiology, such as (a) the natural history of fibrin formation and clearance from the peritoneal cavity and whether addition back of tPA will recover the consequences of the deficiency; (b) the effect of enhancing collagen degradation or the vascularity of the tissues on adhesion formation and to analyse whether reversing these factors had the opposite effect; (c) the effect of decrease of mesothelial cells migration in tPA<sup>-/-</sup> mice in peritoneal adhesion formation; (d) the role and effects of other processes such as MMP activation or looking at PAI levels and their role in adhesion formation; and (e) the type of cells in the peritoneal lavage fluid and the role of each in the clearance or formation of peritoneal adhesions.

It is clear that adhesion formation is a multi-factorial process and more complicated than to single out one mechanism in its evolution, this highlights to us the need for more research to understand the pathophysiology of adhesion formation and to guide us for further therapies to be developed in order to minimize their detrimental effects for the well being of humans.

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## **APPENDIX**

## **A1. HISTOLOGICAL TISSUE PROCESSING**

### **1.1 Fixation**

The primary objective of fixation is to preserve the tissue with the least alteration from the living state. Thus, it must be performed promptly to avoid tissue digestion by enzymes present in the tissues (autolysis). Much of this is attributed to the fact that they coagulate proteins, which are present in great abundance in cells and tissues. The cut adhesion specimens were immediately placed in 4% paraformaldehyde in buffered phosphate saline. This was prepared by the addition of 4 grams of paraformaldehyde to a beaker containing 50 ml of double distilled (dd) water and stirred on a heated hot plate at 65°C. The solution was removed from heat and 1 drop of 10 M NaOH was added. One tablet Phosphate Buffered Saline (PBS) was added, and the solution was made up to 100 ml. with dd. water. The solution was filtered and stored aliquoted at - 20°C. The tissue was fixed in 4% paraformaldehyde overnight.

### **1.2 Wax embedding**

The purpose of embedding is to provide support to the tissue block so that it may be cut into thin sections. Prior to that, the fixed tissue is washed to remove excess fixative and then dehydrated by passing it through increasing strengths of ethyl alcohol or other dehydrating agents, and then to substitute wax for the water initially present in the tissues, so that the tissue block can be sectioned easily. In order to embed the tissues in wax, the specimens were mounted in plastic labeled cartridges and placed in 70% alcohol/dd. water overnight. The cartridges were placed in metal cages, which were mounted in the holder in the wax-processing machine (Shandon Citadel 2000, England). The tissues were automatically processed through various fresh solutions.

The solutions used were:

1. 70% Alcohol
2. 80% Alcohol
3. 90% Alcohol
4. 100% Alcohol
5. 100% Alcohol
6. 100% Alcohol
7. Iso-propyl alcohol
8. Iso-propyl alcohol: chloroform 50:50
9. Chloroform: A clearing agent for the removal of the dehydrating agent and its replacement by some fluid that is miscible both with the dehydrating agent and with the embedding agent.
10. Chloroform
11. Wax
12. Wax

About 24 hours later, the tissues were embedded in wax. A warm metal trough was filled with molten wax. The labeled cartridges were all placed in molten wax using a Tissue - Tek II, Tissue embedding centre. Metal moulds and forceps were heated and the moulds were filled with wax and kept on the hot plate. A cartridge was removed with cold forceps, and the tissue was quickly added to the wax in metal pot in the correct orientation with warmed forceps. The labeled half of the cartridge was placed on the top of the metal mould. After topping up with wax, samples were left to cool on a cold plate for 30 minutes. The wax blocks were stored at room temperature ready for sectioning.

### **1.3 Sectioning**

The wax blocks were kept face down in ice containers before sectioning. Excess wax was chopped from the block with a scalpel and mounted in a chuck in the microtome (Microtome, Pabisch, Germany). Sections were initially cut at 25 $\mu$ m in order to trim the block. When the blade eventually reached the tissues, sections were cut at 5 $\mu$ m. The sections were collected with the aid of a thin paint-brush and forceps and floated on 20% ethanol on a glass plate. The glass plate carrying the sections was gently lowered into the water bath at 40°C and the sections were seen to separate from the glass plate and float on the water. The sections were collected on coated glass slides with 20% poly-1-lysine and allowed to drain. All the slides were labeled with the serial number and the code number. When sectioning was completed, the slides were placed in the oven/heated plate for 30 minutes at 50°C. They were then stored at room temperature ready to be processed with histological stains.

### **1.4 Histological staining**

The purpose of staining is to enhance natural contrast and to make evident various cell and tissue components with an extrinsic material.

#### **1.4.1 Haematoxylin and Eosin (H & E):**

This stain is the most commonly used technique in histology. The basic dye, haematoxylin, stains acidic structures a purplish blue. Nuclei, ribosomes and rough endoplasmic reticulum have a strong affinity for this dye owing to their high content of DNA & RNA respectively. In contrast, eosin is an acidic dye, which stains basic structures red or pink, hence all cytoplasmic structures and intercellular substances are stained pink or pinkish red. Method for (H&E) staining are summarised in Table 6.1.

Table 6.1: Procedure for Haematoxylin and Eosin staining

NO.	PROCEDURE	TIME
1.	Dewax sections in first Xylene	3 minutes
2.	Dewax sections in second Xylene	3 minutes
3.	100% ethanol	1 minute
4.	100% ethanol	1 minute
5.	70% ethanol	1 minute
6.	Running tap water	1 minute
7.	Stain in Harris haematoxylin (regressive stain)	5 minutes
8.	Wash well in running tap water	3 minutes
9.	Differentiate in 1% acid alcohol (agitate)	6 seconds
10.	Wash in Scott's tap water until sections are blue	5 minutes
11.	Wash briefly in tap water	<1 minute
12.	Stain in 1% Eosin	5 minutes
13.	Wash in running tap water	1/2-1 minute
14.	70% ethanol	15 seconds
15.	100% ethanol	30 seconds
16.	100% ethanol	30 seconds
17.	First xylene	1 minute
18.	Second xylene	1 minute
19.	Mount slides in DPX (straight from histoclear)	

#### 1.4.2 Masson's trichrome

Masson's trichrome technique is used to demonstrate supporting tissue elements, principally collagen. Nuclei and other basophilic structures are stained blue, collagen is stained green or blue and cytoplasm, erythrocytes and keratin are stained bright red.

##### Celestine blue solution:

Celestine Blue (Sigma C - 7143)	1.25gm
Ferric Ammonium sulphate from fridge (Sigma F- 1018)	12.5gm
Glycerin	35 ml
Double distilled water	250 ml

The ferric ammonium sulphate is dissolved in the cold dd water with stirring, and the celestine blue is added to this solution and the mixture is boiled for a few minutes. After cooling the stain is filtered and glycerin is added and can be kept up to 6 months.

##### 1% Ponceau Fuschin in 1% acetic acid:

Ponteau fuschin (Masson, Gurr 34186)	5gm
Acetic acid (concentrated)	5 ml
Double distilled water	495 ml

##### 1% P M A

Phosphomolybdic acid (Sigma P-0550)	5gm
dd water	500 ml

##### 0.5% Soluble blue in 2.5% acetic acid

Methyl Blue (water soluble; Sigma M-5528)	2.5gm
Acetic acid	12.5 ml
double distilled water	987.5 ml

Table 6.2: Procedure for Masson's trichrome staining

NO.	PROCEDURE	TIME
1.	Dewax sections in first histoclear	3 minutes
2.	Dewax sections in second histoclear	3 minutes
3.	100% ethanol	1 minute
4.	100% ethanol	1 minute
5.	70% ethanol	1 minute
6.	Running tap water	1 minute
7.	Stain in Celestine Blue	10 seconds
8.	Running tap water	1 minute
9.	Mayers Haematoxylin	10 seconds
10.	Wash in dd water	1 minute
11.	1% Ponceau fuschin in 1% acetic acid	6 minutes
12.	Wash in dd water	1 minute
13.	1% PMA until collagen decolorized	2 minutes
14.	Wash in dd water	1 minute
15.	Counterstain in 0.5% soluble blue in 2.5% acetic acid	45 seconds
16.	Wash in dd water	1 minute
17.	Straight into 100% ethanol	30 seconds
18.	Wash in first histoclear	1 minute
19.	Wash in second histoclear	1 minute
20.	Mount slides in DPX	

## **1.5 Mounting sections**

After removal of the clearing agent, a drop of the mounting medium DPX, which has a refractive index similar to that of glass, is placed on the section. The preparation is covered with a coverslip and allowed to dry. After the mounting medium dries, the specimen is available for microscopic examination and storage.

## **1.6 Microscopic examination and photographing of sections**

The slides were viewed under a light microscope (Axiophot, Zeiss, Germany) and the photographs were taken by using Kodak Ektachrome 64 films.

## **A2. IMMUNOCYTOCHEMICAL TISSUE PROCESSING**

This process is based on the coupling of immunoglobulin to substances that render them visible in the microscope without causing a loss of the antibody's biologic activity. Since the labeled immunoglobulin bind only to their antigens, these compounds permit localization of specific antigens in tissue specimens. Fluorescent dye molecules are chemically linked to antibody molecules, and the sites of their reaction with antigens can be visualised in the fluorescent microscope.

A Silver foil pot was filled with OCT (Tissue Tek ©, Miles Inc. USA) and the adhesion specimen was added making sure that all of the tissue was immersed. The OCT/tissue mould was frozen by placing it on a metal block over liquid Nitrogen. When the OCT/tissue mould became solid white it was placed into a freezer at -80°C ready for cryosectioning. The blocks were removed from the -80°C freezer and kept at -20°C all the times during cryosectioning. In the cryostat (Cryostat MHR, SLEE Technik, Germany), silver foil wrapping was removed and the blocks were mounted on the chuck using OCT. The anti-roll plate was placed on the knife-edge and 7 µm sections were cut and collected on a coated glass slide (2 sections/slide). All the slides were labeled with the serial number



and the code number of the specimen. All the slides were immersed for 10 minutes in ice cold acetone in Coplan jars and allowed to air dry. Slides were then stored at -20°C ready for immunostaining. Slides were washed in PBS and placed in a humidified chamber to reach room temperature. Appropriate serial dilutions of the primary antibody were prepared in Phosphate Buffered Saline (PBS) pH 7.3 and tested to assess the optimal concentration of antibody to be used for the immunolocalisation. When the appropriate concentration of each antibody was determined, 20-50 µl. of the antibody was added to each section, the slides were left for one hour in a sealed humidified chamber. An appropriate dilution of the secondary antibody was prepared and spun for 3 minutes at 13000 rpm. After washing the slides three times with PBS (pH 7.3) 20 - 50 µl. of the secondary antibody was added to each section and the slides were left again in the humidified chamber for one hour. After this period, the slides were again washed 3 times with PBS before mounting. A drop of the mounting media (aqua polymount) was added to each section and covered with a glass cover slip. They were allowed to dry overnight in the dark and stored at 4°C covered with silver foil. The slides were viewed under a microscope with fluorescent filters (Axiophot, Zeiss, Germany) and photographed within a month with a PROVIA Fuji chrome 1600 ASA film using lenses x5, x10 and x20 magnification.

**A3. SUMMARY TABLE DESCRIBING TEMPORAL APPEARANCE OF IMMUNO-REACTIVITY FOR THE DIFFERENT NERVE FIBRE TYPES IN PERITONEAL ADHESION TISSUE**

<b>WEEKS</b>	<b>NEURONAL MARKERS</b>				
	<b>SYN-IR</b>	<b>CGRP-IR</b>	<b>SP-IR</b>	<b>VIP-IR</b>	<b>TH-IR</b>
<b>1 WEEK</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>
<b>2 WEEKS</b>	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>
<b>3-8 WEEKS</b>	<b>++</b>	<b>++</b>	<b>++</b>	<b>++</b>	<b>++</b>

**0:** No detectable immuno-reactive nerve fibres in peritoneal adhesion tissue

**+:** Immuno-reactive nerve fibres were seen in peritoneal adhesion tissue

**++:** More detectable immuno-reactive nerve fibres